Pathogenesis and evolution of Canine Transmissible Venereal Tumour

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Abstract

Canine Transmissible Venereal Tumour (CTVT) is a neoplastic disease occurring naturally in dogs. CTVT is usually sexually transmitted, but transmission may occur by licking, biting, or scratching tumour-affected areas. Cytogenetic studies directed to verify the cell transmissibility of the CTVT showed that the tumour cells are characterized by a rearranged karyotype, which is similar in tumours from in different parts of the world. Thus it was postulated that that the transmissible agent causing the CTVT is the tumour cell itself and that all worldwide CTVT have a clonal origin. Given the lack of a definitive proof of the cellular transmission and clonal origin of CTVT, in this PhD project, I tested this hypothesis by analysing the genetic distance between host and tumour and between tumours, using molecular genetic markers including major histocompatibility (MHC) genes, microsatellite loci, and mitochondrial (mt) DNA in matched tumour and normal tissues from naturally occurring tumours collected worldwide. Here I demonstrate that tumours are genetically distinct from their hosts and that the tumours obtained from 5 different continents are derived from a single neoplastic clone. During its evolution CTVT has diverged into two distinct phylogenetic subclades. Although naturally occurring CTVTs are observed only in dogs, CTVT has been reported to be experimentally transmitted by inoculation of tumour cells in other species of the *Canidae* family such as foxes, coyotes and jackals, thus raising questions about its capacity to grow as an allograft or xenograft and its phylogenetic origin of CTVT. I therefore examined MHC gene transcription in a progressive growing tumour and observed MHC class I and II downregulation. In this study phylogenetic analyses indicate that CTVT most likely originated from a wolf or an East Asian breed of dog. CTVT has been described for the first time in 1876 by Novinsky, thus arguing for an age of at least 130 years old. Here phylogenetic analysis based on microsatellites suggests that CTVT originated between 250 and 2500 years ago.

Contents

List of figures and tables

Glossary

ALT	Alternative lengthening of telomeres
BER	Base excision repair
BLAST	Basic local alignment search tool
BRCA	Breast cancer
CAM	Cell adhesive molecules
CDK	Cyclin dependent kinase
CIITA	Coactivatorclass II transactivator
CIN	Chromosome instability
CML	Chronic myelogenous leukaemia
CR	Control region
CTVT	Canine transmissible venereal tumour
DLA	Dog leukocyte antigens
DP	Difference products
Dps	Proportional shared distance
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
EPPS	*N*-(2-Hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid)
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
HLA	Human leukocyte antigens
HNPCC	Hereditary non-polyposis colon cancer
HVR	Hypervariable region
IAM	Infinite allele model
KGF	Keratinocyte growth factor
LINE	Long interspersed nuclear element
LOH	Loss of heterozygosity
LTR	Likelihood ratio test
MAD	Mitotic arrest deficient
MHC	Major Histocompatibility system
ML	Maximum likelihood
MMR	Mismatch repair
MP	Maximum parsimony
MRCA	Most recent common ancestor
MSI	Microsatellites instability
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
NJ	Neighbor joining
NK	Natural killer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIC	Polymorphism information content
RDA	Representational difference analysis

ROS	**Reactive oxygen species**
RT	**Reverse transcriptase**
SMM	**Stepwise mutation model**
SRY	**Sex determining region**
SSM	**Slip-strand-mispairing**
STR	**Short tandem repeats**
TCR	**Transcriptional coupled repair**
TERT	**Telomeric reverse Transcriptase**
TGF	**Tumour growth factor**
TMRCA	**Time of the most recent common ancestor**
TSG	**Tumour suppressor genes**
UCO	**Unequal crossing over**
VEGF	**Vascular endothelial growth factor**
VNTR	**Variable number of tandem repeat**
XP	**Xeroderma pigmentosum**

Chapter 1

Introduction

1.1 Historical perspective on cancer causation

The term "karkinos" (crab) was employed by Hippocrates as a metaphor to describe a variety of swelling conditions, with finger like spreading projections from a round nodule (Kenneth, 2003). Hippocratic and Galenic medicine attributed the spread of black bile (one of the four humours) in the tissue as the cause of the cancer (Diamandopolus, 1996). This idea survived intact through the Middle Ages and Renaissance. With the discovery of the lymphatic system by Gasparro Aselli in 1662, the black bile theory was superseded by the idea that cancer was an inflammatory reaction to extravasated lymph; a theory modified 150 years later by John Hunter who introduced the notion that contaminated coagulating lymph was the origin of the cancer (Kenneth, 2003). In 1838, German pathologist Johannes Muller demonstrated that cancer is made up of cells and not lymph, but he believed that cancer cells did not originated from normal cells but from a budding element named Blastema (Kardinal and Yarbro, 1979). Following Schleiden and Schwann's cell theory of tissues, it was Rudolf Virchow (Muller's student) who in 1855 that demonstrated that every cell is derived from another cell (omnis cellula e cellula), including cancer cells (Mazzarello, 1999; Porter, 1999). In 1867 Wilhelm Waldeyer supported the theory of the normal cell for the origin of cancer and he believed that metastasis resulted from transportation of cancer cells by blood or lymph (Porter, 1999).

Around the turn of the twentieth century the beginning of tumour transplantation experiments led to the new view of the cancer cell as an autonomous cell. The first successful tumour transplants were described in 1876 by the Russian veterinarian Mstislav Aleksandrovich Novinski (Novinski, 1876). He reported in his thesis

entitled *On the Question of the Inoculation of Malignant Neoplasms* the first successful serial passage of tumours through transplantation in dogs. Novinski's transplantation experiments were based on the inoculation of canine transmissible venereal tumour (CTVT) in puppies. Novinski stated that successful tumour transplantation depends on the inoculation of a living element of the tumour and that the transplantation of the element of a cancerous tumour to healthy tissue acts as an infecting agent. In 1888 Wehr repeated Novinski's transplantation experiments in dogs with similar results (Shimkin, 1955). It is interesting to note that the dogs used for transplantation of CTVT did not come from a single breed and were therefore not highly inbred. The allo-transplantation of tumours seemed less surprising in the late 19th Century than it does today with our modern knowledge of histo-incompatibility.

The successful results obtained with CTVT served as model for tumour transmission in other animals. Hanau in 1898 inoculated two rats with vulvar epidermoid carcinoma and observed growth of the tumour in the recipients (Shimkin, 1955). In 1901 Leo Loeb supported the transplantability of tumours in rats (Witkowski, 1983; Brent, 1997). In 1903 a Danish veterinarian Carl O. Jensen demonstrated that the successful growth of transplanted tumours in mice is determined by heredity (Brent, 1997). The discovery that the tumour could be successfully transplanted into other mice, led to the idea of using the rodent system to supply tumours for experiments. The observation that a single tumour can be expanded through many generations exceeding the life span of the laboratory mouse led Leo Loeb to the "cancer immortality" concept (Witkowski, 1983).

One of the most important steps in the discovery of the mechanistic basis of the origin of cancer is due to Theodor Boveri (Allan, 2001). The results of his experiment on the fertilization of the *Ascaris megalocephala* eggs first and *Paracentrotus* sea urchin later led to Boveri in 1902-1904 to postulate the chromosomal theory of heredity (Baltzer, 1964). The observation that the sea

urchin eggs carrying an abnormal number of chromosomes showed abnormal growth, and the observation made by David Von Hansemann of abnormal mitosis in tumour cells, led Boveri in 1914 to develop the theory that the malignant tumours originated by incorrect chromosome segregation (genome instability) and that this abnormality was passed to daughter cells (clonal origin (Allan, 2001; Rowley, 2001).

The earliest observations reported by John Hill in 1759 and by Percival Pott in 1775 on the association of a specific tumour to a specific profession or work, led to the idea that some chemicals can cause cancer (Greaves, 2000). In 1918 Yamagiwa and Ichikawa induced cancer by applying coal tar to rabbit skin (Greaves, 2000; Luch, 2005).

After the discovery of the X rays by Wilhelm Conrad Roentgen in 1895, Frieben published data in 1902 indicating that cancer rates were increased among persons working with X-rays (Cassileth, 1983; Greaves, 2000)

The concept that cancer might be contagious also recurs throughout the past 300 years. In the XVII and XVIII centuries, physicians Daniel Sennert and Zacutus Lusitanus supported the hypothesis that cancer was contagious. In fact in 1779 a hospital in Paris dedicated mainly to cancer patients was forced to move from the city because of fear of cancer spread to the population in the city (Cassileth, 1983; Kenneth, 2003). In 1844 the Italian physician Domenico Antonio Rigoni-Stern noted that cancer of the cervix was frequent among married ladies, rare among unmarried ladies and absent in Italians nuns. In contrast, breast cancer was more frequent among nuns (Greaves, 2000). These observations led to the hypothesis that cervical cancer was sexually transmitted, and we now know that the cause is a papilloma virus (zur Hausen, 2002). In 1908 Wilhelm Ellerman and Olaf Bang, studying leukaemia in chickens succeeded in transferring the disease from one animal to another by cell-free tissue filtrates (Wyke, 2003). In 1911 Peyton Rous

demonstrated that viruses were the cause of solid tumours (Sarcoma) in chickens but it took many decades before his data were accepted (Dulbecco, 1976). The notion that viruses can cause cancer was a discovery that brought back the fear that cancer was a contagious disease.

With the discovery made by Hermann Muller in 1930, that exposure to X rays causes mutations in fruit flies, cancer scientists began a series of experiments to find the causal role of genetic alteration in cellular transformation (Weinberg, 2001). One of these experiments was made in the laboratory of Michael Bishop and Harold Varmus (Stehelin et al., 1976) with the demonstration that normal chicken DNA contains a homologue of the viral gene v-*src* oncogene of Rous Sarcoma Virus. V-src is able to transform normal cells and the homologus sequence was present in all normal cells as a proto-oncogene. Robert Weinberg, and Michael Wigler, using the DNA transfer approach, demonstrated that DNA extracted from chemically induced tumours was able to induce transformation when transferred in to a normal cell (Shih et al., 1979; Perucho et al., 1981). This observation was soon confirmed with human tumours (Perucho et al., 1981). When the transforming DNA was cloned and sequenced, it was recognized as a mutated form of the k-*ras* gene, a gene first characterized in oncogenic retroviruses (Shimizu et al., 1983). These new discoveries had a profound effect in converging all the causation theories into a unified theory that sees the mutation of DNA of specific genes as a key target in carcinogenesis.

1.2 Tumour Progression

The first detailed characterization of the dynamic nature of cancer was described by Leslie Foulds (Foulds, 1949). Foulds showed that tumours progress (evolve) through different stages, characterized by the acquisition of different phenotypic traits such as increased growth rate, hormone dependence, invasiveness, formation of metastasis (Foulds, 1949; Foulds, 1954; Foulds, 1957). With the progress of

molecular biology the phenotypic view has been replaced with the somatic mutation theory, where cancer evolves through the accumulation of different mutations in several genes (Greaves, 2000). The accumulation of mutations in somatic cells implicates the presence of different cells bearing different mutations and also the presence of natural selection, which selects the cells with advantageous mutations. One of the questions arising from the somatic mutation theory is whether a tumour has a single or a multiple origin.

To determine mono- or poly-clonality of the tumour, Philip Fialkow used the X linked glucose 6-phosphate dehydrogenase (G-6-PD) gene as a genetic marker (Linder and Gartler, 1965; Fialkow et al., 1967). In females one of the two chromosomes X are randomly inactivated through methylation, therefore only genes present in the non-methylated X chromosome are expressed in a given somatic cell. The human population, especially African Americans, has two frequent G-6-PD variants called A and B. Therefore in accordance to the random X inactivation, in females heterozygous for G-6-PD we expect to have two bands with different electrophoretic mobilities. Analysis conducted in G-6-PD heterozygous female tumours indicate that in all tumours analyzed, the tumours were monoclonal (Fialkow, 1979).

This observation was supported by a karyotype analysis in chronic myelogenous leukaemia (CML) by Peter Nowell and David Hungerford in 1960 (Nowell, 2002). They described the presence of an unusually short chromosome 22 in all CML tumour cells analyzed, and the absence in the normal cells from the same patients. This observation suggested that this mutation was a somatic mutation that occurred in one cell in the bone marrow, which gave it a selective advantage to expand as a clone. Nowell postulated that a tumour develops by a Darwinian evolutionary process, where cells with mutations conferring a growth advantage are selected and expanded (Nowell, 1976; Greaves, 2002).

Although the notion that cancer evolved in a multistep fashion has been determined by applying molecular biology techniques, the notion that cancer progresses through several events has been suggested during the 1950s by applying mathematical models (Weiss, 2004). In 1949 Isaac Berenblum and Philip Shubik developed the two stage model, which was based on the observation that cancer can be induced by the application of mutagens followed by a non-mutagenic promoter, such as phorbol ester (Berenblum and Shubik, 1949). In 1954 Peter Armitage and Richard Doll analyzed human cancer incidence over the age, and showed that the incidence of cancer increased with the sixth of power of age (Armitage and Doll, 1954). They postulated the multistage theory of cancer and inferred that before the clinical manifestation of cancer, the cellular lineage needs 6-7 independent sequential steps of transformation (Armitage and Doll, 2004). Based on the observation of the incidence of Retinoblastoma in children, Alfred Knudson postulated the two hit hypothesis (Knudson et al., 1975). Retinoblastoma is a tumour that becomes manifest early in life. Retinoblastoma can be inherited or sporadic. According to the two hit hypothesis in the inherited form a single mutation in the Retinoblastoma (Rb) gene is present in the germ line which gives the genetic predisposition to develop cancer, but a second mutation in the normal Rb allele which occurs in the retinoblast must be acquired to develop cancer (Knudson, 2001). In the sporadic form the two mutations in the Rb alleles occur in the somatic cells. Although the epidemiological and molecular observations have consolidated the multistage theory of cancer, the number of mutations and in which sequential order they have to be acquired to develop cancer is still an open question (Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002b).

Early experiments involving transforming retroviruses and the transfer of genes from tumour cells into established rodent cells allowed the identification of several cancer causing genes called oncogenes. The result of these experiments suggested that cancer could be induced by the mutation of one proto-oncogene. However the rodent cells used as recipient in the gene transfer experiments were not normal, but were immortalized, thus acquiring the ability to proliferate indefinitely. When the

normal rodent cells were used, the transfer of a single oncogene failed to induce transformation, while the transfer of two oncogenes resulted in transformation. Recent evidence suggested that human cells require more mutations than rodent cells and that there are differences also between cell types within the same species (Rangarajan et al., 2004).

1.3 Cancer Hallmarks

Despite the enormous variety of tumours affecting different types of tissues in animals and humans, research over the past 50 years has revealed that all malignant cancer share the same essential alterations (Hanahan and Weinberg, 2000). These alterations are the result of breaching specific barriers evolved by multicellular organisms through their evolution from unicellular organisms (Michod, 2002). These alterations include:

Immortalization

Evasion of programmed cell death (apoptosis)

Independence from growth stimulation

Resistance to growth inhibition

Angiogenesis

Invasion and metastasis

Genetic instability.

These hallmarks are briefly described below.

1.3.1 Immortalization

Early experiments performed by Hayflick in 1961 demonstrated that normal cells when propagated in culture possess a limited replication capacity (Hayflick and Moorhead, 1961). The replicative capacity differs between cells within species and between species (Hahn and Weinberg, 2002a). When cells reach the limit of division a specific cellular program called senescence is activated. Since senescence

limits the number of cellular divisions it plays an important role in aging and tumour biology (Mathon and Lloyd, 2001; Hahn and Weinberg, 2002a). The observations that DNA polymerase is unable to copy the extremity of the chromosomal DNA called the telomere, thus shortening the telomere during each mitosis, led Olovnikov in 1970 to propose that since the mammalian cells are unable to duplicate the telomeres, during mitosis the telomere are eroded until the chromosome ends are fused, causing genomic abnormalities which result in cellular death (Olovnikov, 1973; Olovnikov, 1996). Over the past 25 years the results of numerous experiments have supported Olovnikov's speculations.

Telomeres contain DNA sequence repeats and protein. The repeat sequence consists of hexameric motifs such as GGGTTA in humans, extended for 10 –20 kilobases. The 3' end has a 100-400 nucleotide over-hang (Mathon and Lloyd, 2001). Telomeric DNA is generated by a reverse transcriptase called telomerase, containing a RNA subunit and a catalytic protein subunit, Telomerase Reverse Transcriptase (TERT). The telomerase RNA binds the telomeres DNA ends thus acting as template for telomere elongation. The chromosome ends are protected by several proteins: TRF-1, TRF-2, and POT–1 (Mathon and Lloyd, 2001; Hahn and Weinberg, 2002a). Several experiments have shown that senescence is activated when the telomeres are shortened down to 5 kb and that senescence is triggered by the shortest telomere present in the cell (Hemann et al., 2001).

Recent reports have suggested that the replicative senescence is not activated by the erosion of the double strand repetitive sequence, but by the degradation of the 3'end single strand overhang, resulting in loss of protective capping (Stewart et al., 2003). Telomere length is maintained by the activation of telomerase or by an alternative mechanism called alternative lengthening of telomeres (ALT), where the telomeres are regenerated through recombination-based interchromosomal exchange of sequence information (Bryan et al., 1997; Dunham et al., 2000). In the normal cell telomerase is transiently expressed, since it can be detected only in S phase, but in

neoplastic cells its expression is increased and is detectable throughout the cell cycle (Mathon and Lloyd, 2001).

Although ectopic expression of telomerase in fibroblast cells results in increased replication capacity (Bodnar et al., 1998), recent experiments reveal that not all cells can be immortalized by telomerase activity (Kiyono et al., 1998) and that senescence is not an intrinsic mechanism independent from extrinsic stress, but a protective mechanism that can be activated also by external factors such as oxygen and metabolic stress, and internal factors such as the overexpression of oncogenes, that occur in cells with normal telomere length (Ben-Porath and Weinberg, 2004). Senescence can be triggered by the activation of the tumour suppressor proteins p53 and or Rb (Hahn and Weinberg, 2002a). While telomere erosion is detected as double strand breaks, thus leading to the activation of the ATM/ATR and or p14/arf complexes, which activate the p53 cascade, the external stress activates the p16 protein causing activation of the Rb pathway (Mathon and Lloyd, 2001). The inactivation of p53 or Rb leads cells to bypass the senescence barrier with further erosion of telomeres, until the cells reach a second proliferative block known as the crisis barrier, activating programmed cellular death (Reddel, 2000).

In tumour cells the senescence and crisis barriers are avoided by the activation of telomerase regenerating the telomeres and by the inactivation of tumour suppressor and pro-apoptotic genes (Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002b).

1.3.2 Apoptosis.

Multicellular organisms are organized in tissues arranged to form organs. Behind this organization there are highly regulated processes acquired through evolution from unicellular to multicellular organisms (Michod, 2002). One of these processes, known as programmed cell death or apoptosis, plays a key role controlling the

number of cells during development and adult life by inducing the superfluous and defective cells to commit suicide (Alberts et al., 2002i).

In adult tissues the number of cell divisions is balanced by cell death. In cancer this equilibrium is shifted toward proliferation. The apoptosis process is organized by sensors and effectors (Hanahan and Weinberg, 2000). The sensors detect the intra- and extra-cellular signals. The intracellular signals include DNA damage, hypoxia and oncogene overexpression (Evan and Littlewood, 1998). The extracellular signals monitor the cell-cell and cell-matrix homeostasis (Aoshiba et al., 1997; Alberts et al., 2002i; Prince et al., 2002). The signals detected by the sensor are mainly conveyed to the mitochondria, where a series of cytoplasmatic proteins of the Bcl2 family control the release of cytochrome C from the mitochondria (Alberts et al., 2002i). The release of cytochrome C activates an array of intracellular proteases called caspases causing protein and DNA degradation (Hanahan and Weinberg, 2000). The caspases can be directly activated by extracellular proteins such as FAS ligand, which binds to the death receptor FAS (Houston and O'Connell, 2004).

Once the caspase cascade is triggered it cannot be inactivated (Alberts et al., 2002i). It has been reported that the tumour suppressor p53 can trigger the caspase cascade by the overexpression of the Bax protein, a member of the Bcl2 family, which in turn increases cytochrome C release thus inducing apoptosis (Hanahan and Weinberg, 2000).

It has been observed that in over 50% of human tumours, the tumour suppressor p53 gene is functionally altered. p53 plays a key role in the maintenance of the genome integrity (Harris, 1996). From these considerations it has been suggested that when the level of DNA damage exceeds the DNA repairing capacity, p53 activates the apoptosis pathway, thus avoiding the replication of mutated cells. In cancer, p53 inactivation allows the neoplastic cell to proliferate with a mutated genome (Levine,

1997). A number of genes encoding proteins involved in inhibiting or promoting apoptosis are found to be mutated in many different kinds of cancer (Lowe and Lin, 2000).

In CTVT, the subject of this thesis, it is likely that expression of c-*myc* is up-regulated, due to insertion of a LINE-1 element as discussed later. Ectopic c-myc expression can promote tumour growth and survival, as seen, for instance, in immunoglobulin gene c-*myc* chromosome rearrangements in Burkitt's lymphoma (Hemann et al., 2005).

1.3.3. Independence from growth stimulation

In complex, multicellular organisms, cells cooperate with each other for the survival of the whole organism. At the core of the cooperation there is the dependence of a single cell on other cells, so that cells are dependently evolving units (Lachmann et al., 2002). Normally the passage from the quiescent to the proliferative state requires the presence of mitogenic growth signals in the microenvironment surrounding the cells. Thus the proliferation of a cell is dictated by the needs of the cells around it (Hanahan and Weinberg, 2000). In contrast, a tumour cell escapes from the external dependence to become an autonomous evolving unit, by producing its own growth signals (Hanahan and Weinberg, 2000).

Although the proliferative stimuli that are exerted by soluble growth factors generally act in a paracrine fashion, where growth signals produced from one type of cell are able to stimulate a different type of cell, a tumour cell may produce growth factor that activates itself (autocrine stimulation) generating a positive feedback (Reith and Panayotou, 2003). One of the growth factors produced by many tumours is platelet–derived growth factor (PDGF) that interacts with the PDGF receptor present on the tumour cell surface which in turn activates the

transcription of c-*myc* and c-*fos* oncogenes (Rozengurt et al., 1985; Benito and Lorenzo, 1993; Pietras et al., 2003).

Another mechanism selected by tumour cells is the overexpression of growth factor receptors, which induce the tumour cells to become sensitive to concentrations of growth factor that normally do not trigger proliferation (Hanahan and Weinberg, 2000). Proliferation can also be induced by a mechanism independent of the growth factor, for example the alteration of the cytoplasmic tail of the epidermal growth factor receptor (EGFR) causes self-activation of the receptor, which therefore becomes independent from the external microenvironment (Alberts et al., 2002a).

The role of the external microenvironment in regulating cellular proliferation is progressively gaining evidence. Cells are connected with the extracellular matrix by receptors called integrins (Alberts et al., 2002h). Integrins not only attach cells to their external environment, but also activate intracellular signalling pathways in response to the extracellular matrix (Alberts et al., 2002h). The association between integrins and extracellular matrix can also be regulated by intracellular signals (Giancotti and Ruoslahti, 1999). Tumour cells are able to modify the relative expression of specific integrins that promote cellular proliferation (Danen, 2005).

Although tumour cells are generally described as independent evolving units, recent results suggest that tumour cells are able to stimulate stromal cells to produce growth factors that increase tumour proliferation (heterotypic stimulation) (Kinzler and Vogelstein, 1998; Skobe and Fusenig, 1998; Iyengar et al., 2003). It has been demonstrated that cells involved in the immune response to tumours may produce factors such as inflammatory chemokines that may also promote the tumour proliferation (Pollard, 2004; Wyckoff et al., 2004).

1.3.4 Resistance of growth inhibition

To balance the activity of the growth signals, the multicellular organism possesses several mechanisms that regulate proliferation. Like growth signals, the anti-proliferative signals derive from soluble factors or surface proteins that are produced by neighbouring cells, or are induced by components of the extracellular matrix (Hanahan and Weinberg, 2000; Alberts et al., 2002d). These external inhibitory signals activate different intracellular pathways that regulate the cell cycle (Alberts et al., 2002b).

The cells within a multicellular organism can be found in three different states: quiescent, proliferative, and post-mitotic (Alberts et al., 2002g). Normally the quiescent and proliferative states are reversible, whereas the post-mitotic status is irreversible (Alberts et al., 2002g). It is thought that the majority of the anti-proliferative signals act in the transition from the cellular G1 to S phase of the cell cycle (Hanahan and Weinberg, 2000).

The Rb protein and its related proteins, p107 and p130, play a key role in controlling this transition (Weinberg, 1995). The association of Rb with the transcription factor E2F inhibits the transcription of genes involved in the G1-S progression (Alberts et al., 2002b). The hyper-phosphorylation of the Rb protein induces the dissociation with E2F, therefore allowing progression to S phase (Alberts et al., 2002b). Normally complexes of cyclin and cyclin dependent kinase (CDK) induce the phosphorylation of the Rb protein (Alberts et al., 2002b). Many tumours can avoid the antigrowth signals by altering Rb activity or the proteins involved in Rb phosphorylation (Mittnacht, 2005).

Retinoblastoma and osteosarcoma are examples of tumours where Rb activity is altered (Mittnacht, 2005). Some glioblastomas and breast cancers are found to have

the CdK4 or cyclin D1 genes amplified, therefore increasing Rb phosphorylation and consequently cell proliferation (Alberts et al., 2002a).

1.3.5 Angiogenesis

All cells within tissues and organs are supplied with oxygen and nutrients by the vasculature system. During development cellular growth is coupled with the formation of new blood vessels (Cleaver and Melton, 2003). In adult tissues, new vessels are required in response to tissue damage and tissue remodelling (Carmeliet, 2003). The small blood vessels consist of only endothelial cells, whereas in larger vessel, pericytes or smooth muscle cells surround the endothelial cells (Carmeliet, 2003). Although it is commonly believed that blood vessels in different tissues consist of endothelial cells with identical properties, recent results suggest that different organs are equipped with distinct and specialized endothelial cells (Cleaver and Melton, 2003). Although the majority of the new vessels in adult tissues are derived by sprouting from existing vessels, recent evidence indicates that progenitor endothelial cells are derived from the bone morrow contributing to the vessel growth (Zhang et al., 2002; Contreras et al., 2003; Nishimura and Asahara, 2005; Religa et al., 2005). The formation of new vessels is referred as vasculogenesis, when the endothelial cells are derived from endothelial progenitors to form a primitive vascular bed, and as angiogenesis and arteriogenesis, when new vessels result from the expansion of existing vessels (Carmeliet, 2003). Although endothelial cells are highly proliferative in response to several angiogenic factors, they have long half-lives up to several years (Carmeliet, 2003). Generally, all cells residing within 100 μm of a capillary blood vessel have access to oxygen and nutrients by diffusion, but when a new tissue grows beyond the diffusion limit, the formation of new vessels is triggered (Hanahan and Weinberg, 2000). In order to adapt the vascular system to the tissue's requirements, several mechanisms regulate the process of angiogenesis (Carmeliet, 2003).

Numerous observations indicate that small tumours (less than 1 mm) can persist for a long period of time (from months to years) in an avascular, quiescent state (Folkman, 2002). Thus in order to grow, tumour cells must change the local equilibrium between positive and negative regulators of angiogenesis. Several studies demonstrated that a tumour acquires the ability to form its own vasculature system in mid-stage lesion, thus indicating that angiogenesis is essential for rapid clonal expansion (Hanahan and Weinberg, 2000). A key molecule involved in the angiogenesis process is the vascular endothelial growth factor (VEGF) (Carmeliet, 2003). Tumour cells are able to promote angiogenesis by over-expressing VEGF or by down-regulating angiogenic inhibitors like thrombospondin or beta-interferon (Folkman, 2002). In addition it has been demonstrated that tumours can activate or inactivate pro- and anti-angiogenic factors respectively present in the extracellular matrix by producing several proteases (Gately et al., 1997; Harlozinska, 2005). Recently it has been suggested that different organs and therefore different tumours use different promoting and inhibitory factors to trigger the formation of new blood vessel (Harlozinska, 2005).

1.3.6 Metastasis

The majority of the cells in multicellular organism are bound and organized in tissues and organs. Cells are held together indirectly by extra-cellular matrix and directly by cell-cell junctions (Alberts et al., 2002c). The extra-cellular matrix is composed of proteoglycans and fibrous proteins such as collagen, elastin, fibronectin and laminin (Alberts et al., 2002c). While the proteoglycans form a hydrated gel substance, the fibrous proteins have structural and adhesive functions. Cells are bound to the extra-cellular matrix and to their neighbours by several types of adhesion molecules; while transmembrane adhesion proteins called integrins mediate the interaction between cells and extra-cellular matrix, the transmembrane proteins that belong to the cadherin family mediate the interactions between cells (Alberts et al., 2002c). Although cell adhesion molecules have an essential structural

role, they are also involved in the communication network between the extra- and intra-cellular microenvironment. While the majority of cells are immobile, cellular migration plays an important part during embryonic development, in adult tissue repair processes and in immunological processes. Cells of the haematopoietic system including tissue macrophages (or histiocytes) are naturally mobile (Imhof and Aurrand-Lions, 2004). In cancer during tumour progression, some tumour cells acquire the ability to migrate and form new colonies at secondary sites (Hanahan and Weinberg, 2000). Metastasis is the migration of tumour cells from their primary tumour formation site to secondary sites where they invade normal tissue and proliferate to form new tumours (Hanahan and Weinberg, 2000). It has been estimated that 90 % of mortality associated with cancer is due to metastasis (Sporn, 1996). Recent results show that few cells in the primary tumour acquire the ability to grow in the secondary sites and that the tendency to metastasise is acquired in the early steps of tumour progression (Van't Veer and Weigelt, 2003).

The ability of tumour cells to spread in new tissues or organs relies on overcoming a series of interdependent steps, which include: cell detachment, motility, local invasion, intravasation, circulation, adhesion, extravasation, proliferation and growth (Nguyen, 2004). In order to migrate, the tumour cell must alter the cell-cell and the cell-matrix interactions (Zigrino et al., 2005). It has been shown that in many tumours the transcription of E cadherin is reduced (Christofori and Semb, 1999; Bogenrieder and Herlyn, 2003). This reduction can be induced by genetic or epigenetic events (mutations or transcriptional repression), increased proteolysis, and redistribution to a different cellular site (Bogenrieder and Herlyn, 2003). Another mechanism adopted by tumour cells is to switch the expression of cell adhesive molecules (CAMs) with high adhesive capacity to CAMs with poor adherence capacity, thus favouring cellular mobility (Hanahan and Weinberg, 2000; Bogenrieder and Herlyn, 2003). An essential step in the metastatic process is the degradation of the extra cellular matrix by the proteolytic activity of proteases produced directly by tumour cells, or indirectly by stromal or immune cells (Egeblad and Werb, 2002). The increase of protease activity can be due to up-

regulation of protease expression, down regulation of the expression of protease inhibitors and increased conversion of active forms (Egeblad and Werb, 2002).

Cell motility is a complex and highly coordinated process, therefore is likely that tumour cells acquire motile ability by altering the expression of genes implicated in the motility process (Bogenrieder and Herlyn, 2003). Although gene expression comparison between metastatic and non metastatic tumours suggest the requirement of cell-intrinsic changes to confer the motile competency (Condeelis et al., 2005; Wang et al., 2005), other studies suggest that extrinsic factors present in the microenvironment are also required to trigger the motility responses (Zigrino et al., 2005).

Progressive alteration of normal tissue homeostasis by tumour and stromal cells, allow tumour cells to move throughout degraded matrix, and to invade surrounding tissues (Hanahan and Weinberg, 2000). Tumour cells are also aided to migrate by soluble factors (chemotaxis) and bound adhesion molecules (haptotaxis) (Nguyen, 2004).

Tumour invasion can be also due to mechanical pressure induced on the nearest tissues by rapid tumour growth causing local tissue damage, thus favouring the tumour invasiveness (Hart, 2003). Progressive local invasion allows tumour cells to invade the systemic circulation (Hart, 2003). Although tumour cells may acquire the ability to form new blood or lymphatic vessels, tumour cells may invade a pre-existing vessel or invade directly the blood vessel, or may penetrate first the lymphatic vessels and then invade the haematogenous system (Nguyen, 2004). Essential to this end is the ability of tumour cells to recognize different adhesive molecules present in lymphatic and haematogenous systems (Dua et al., 2005).

In order to invade new organs, circulating tumour cells need to stop and exit the systemic circulation. In an unspecific manner, the extravasation may be due to the

fact that large arteries progressively narrow in to arterioles and then capillaries, and tumour cells can be trapped in this small vessel, thus allowing the migration in the new organ (Nguyen, 2004).

Although tumour cells may invade several organs, it has been shown that certain types of cancer form metastases in specific organs. In 1889 Stephen Paget suggested that the colonization of a new organ by tumour cells is the result of the interaction of circulating tumour cells (seed) and the target tissue (soil) (Fidler, 2003). Although the exact mechanism behind the tumour homing is not completely understood, recent results suggest that the selective homing of cancer cells may be due to three mechanisms: 1) presence in the target tissue of specific growth factors or appropriate extra-cellular matrix that favour the selective tumour growth, 2) presence in the target organ vessel endothelium of specific adhesive proteins that interact with the tumour cells, favouring the tumour invasion, 3) production of a chemotaxis soluble factor by the target tissue that attract the tumour cells (Fidler, 2003). Recently it has been reported that tumour cells by secreting specific humoral factor(s) can mobilize haematopoietic bone marrow progenitors cells to specific organs, where they change the local environment to form a pre-metastatic niche that attracts and supports the incoming tumour cells thus favouring the development of metastasis (Kaplan et al., 2005).

Recently it has been shown that the gene expression pattern between tumour cells in primary and secondary tumours are similar, thus suggesting that the metastatic ability is acquired in the early steps of the tumour progression, and that the genetic alterations responsible for tumorigenesis are involved in the metastatic process (Bernards and Weinberg, 2002; Van't Veer and Weigelt, 2003).

1.3.7 Genetic instability

Over the past 25 years numerous genetic alterations have been described in human and animal tumours. These genetic alterations can affect the DNA sequence and the chromosomes (Lengauer et al., 1998). The mutations of DNA include: substitution, deletion, translocation and insertion and they can affect one or more nucleotides. Although genetic variation is considered fundamental for evolution, the necessity to transmit genetic information faithfully between generations demands genetic stability (Eisen and Hanawalt, 1999). These two opposing demands are accomplished by controlling the genetic variation rate. In normal conditions the genome is affected by spontaneous mutations caused by physiological DNA instability and by imprecision of the DNA polymerase proofreading activity during the DNA replication (Alberts et al., 2002e).

DNA polymerases limit nucleotide misincorporation by two mechanisms: one mechanism acts before nucleotide incorporation because the DNA polymerase conformation changes when a nucleotide is misincorporated causing the dissociation between the polymerase and DNA (Alberts et al., 2002f); the second mechanism, known as exonucleolytic proofreading acts after nucleotide misincorporation. DNA polymerase has two catalytic sites; the first is called the polymerization site and is active when the enzyme is adding the correct nucleotide in the 5' to 3' direction during DNA replication; however, once the incorrect nucleotide is covalently added, the nascent 3' end is moved to the second site, called the exonucleolytic site, where the unpaired nucleotide is removed, allowing to shift the growing strand back to the polymerization site where the correct nucleotide is then added (Alberts et al., 2002f).

In eukaryotic cells, several enzymes have been described with DNA polymerization activity, and five are the most important DNA polymerases involved in DNA replication and repair: α (alpha) β (beta) γ (gamma) δ (delta) and ε (epsilon). To date

the only polymerase involved in mitochondrial DNA replication is polymerase gamma. In vitro studies on the fidelity of DNA duplication has shown that the nucleotide misincorporation rate varies among polymerases, with one in 5000 bases for beta and one in 10 000 000 for delta an epsilon polymerases (Umar and Kunkel, 1996; Loeb and Loeb, 2000). To avoid non-complementary nucleotide incorporation, polymerase delta, gamma and epsilon contain a proofreading activity (Kunkel and Alexander, 1986). Normally DNA replication is carried out by delta polymerase, but recent reports show that in some tumours this priority is shifted in favour of less accurate polymerases, thus increasing the mutation rate (Loeb and Loeb, 2000). Environmental agents such as ultraviolet light, ionizing radiations and toxic substances in the dietary uptake can induce mutations (Loeb and Loeb, 2000).

Spontaneous mutations also occur in the normal cellular environment, due to depurination and deamination processes and also to the formation of reactive oxygen species generated from the mitochondrial electron transfer process (Alberts et al., 2002e). In order to repair the mutated DNA, cells have evolved several repair mechanisms, which involve different pathways depending on the type and extension of mutation.

1.3.7a Base excision repair

The base excision repair (BER) pathway is involved when a mutation affects a single nucleotide. BER employs enzymes called DNA glycosylases, which are specific in removing a specific mutated base (Krokan et al., 2000). The enzyme recognizes the altered base through a base flipping mechanism. Once the mutated base is recognized the enzymes hydrolyze the base-sugar linkage leaving a sugar without a base. This sugar is then recognized by an apurinic/apyrimidinic (AP) endonuclease and phosphodiesterase which remove the sugar from the

phosphodiester backbone. The missing nucleotide is replaced by DNA polymerase beta and linked to the next nucleotide by DNA ligase I or III (Krokan et al., 2000).

1.3.7b Nucleotide excision repair

The nucleotide excision repair (NER) system is able to repair DNA damage induced by UV. In contrast to BER, the NER system recognizes altered nucleotides by scanning the DNA for a conformational alteration (bulky lesion) (Wood, 1996). The human NER system consists of several proteins called XP (labelled XP-A through XP-G) because they were identified by studying the defective repair process in individuals affected by Xeroderma Pigmentosum (Cleaver, 1968). This is a rare autosomal recessive disorder, characterized by mutation in at least one of the XP genes, which give a marked genetic predisposition to develop skin cancer (Cleaver, 1968).

The repair process begins with the detection of DNA conformational alterations by the XP-C protein (Wood, 1996). The XP-C then recruits the transcription factor TFIIH, which subsequently interacts with XP-A protein and RPA proteins. This complex then interacts with the helicases XPB and XPD to unwind the double stranded DNA. Subsequently the 3'endonuclease XPG and XPF cut out from the altered region 2-9 or 16-25 nucleotides respectively. The new 3 free termini interact with PCNA and RFC proteins, which in turn allow DNA polymerase delta to fill the gap. The process is then completed by DNA ligase I.

Although the NER system is involved in repairing the total genome, NER seems to act more efficiently on transcriptionally active genes. This activity called transcriptional coupled repair (TCR), guarantees the genetic integrity of the most

essential genes (Hanawalt et al., 2003). Recent observations suggest that TCR is also present in BER and mismatch repair pathways (Leadon, 1999).

1.3.7c Mismatch repair

The mismatch repair (MMR) pathway includes a series of proteins that are involved in correcting errors that escape the DNA polymerase proofreading activity during DNA replication. They are also involved in suppressing recombination between non-identical sequences both in mitosis and meiosis (Kolodner and Marsischky, 1999). Unlike BER and NER, MMR does not act on damaged or mutated sequences, but it targets only the newly synthesized DNA strand. In eukaryotes several MMR proteins have been described that are homologous to those characterized in prokaryotes and therefore have an ancient lineage (Kolodner, 1996; Eisen and Hanawalt, 1999).

The repair process commences with the recognition of the DNA mismatch region by a complex formed by the interaction of hMSH2 with hMSH6 or hMSH3 proteins (Kolodner, 1996; Kolodner and Marsischky, 1999). This heterodimer undergoes an ATP dependent conformational change, which enables it to move along the DNA backbone. The hMSH2/hMSH6 complex recruits a second complex composed of hMLH1 and hPMS2. This ternary complex is able to move in both directions until it encounters the PCNA protein, which guides the MMR complex to the 3' free DNA termini present in the newly synthesized strands. At this stage the MMR complex activates an exonuclease able to degrade the newly synthesized strand including the mismatched region, thus removing the MMR complex from the mismatched sequence. The sequence gap is then filled by the DNA polymerase delta (Kolodner, 1996; Kolodner and Marsischky, 1999). It has been reported that the MMR system decreases the level of replicative errors down to 100-1000 fold (Modrich and Lahue, 1996). The importance of the MMR activity has been also highlighted by the fact that individuals with inherited mutation of MMR loci are genetically predisposed to

develop colon cancer (Lengauer et al., 1998). The most common form of colon cancer predisposition is the hereditary non-polyposis colon cancer (HNPCC), where mutations in MMR genes present in the germ line (MLH1 and MSH2 are the most common) are predisposed to develop colon cancer if a somatic mutation affects the normal allele with the Rb system (Lengauer et al., 1998). Given that MMR is important for repairing errors affecting repetitive sequences, inactivation of the MMR system produces microsatellite instability (MSI) (Atkin, 2001).

1.3.7d Recombinational repair

Double strand breaks (DSBs) are commonly caused by ionizing radiation, however they also occur during the rearrangement process of the immunoglobulin and T cell receptor genes. In eukaryotes, two processes called homologous recombination (HR) and non-homologous end joining (NHEJ) can repair double strand breaks. Recent studies have shown that both processes can act simultaneously or alternatively and that their relative contribution varies during the cell cycle and development (Essers et al., 2000; Haber, 2000).

1.3.7e. 1 Homologous recombination

Homologous recombination repairs double strand breaks by using an intact and homologous DNA molecule as a template. In eukaryotes several proteins are involved in the homologous recombination process (Kanaar et al., 1998; Haber, 2000). Although Rad51 has a central role in the recombination process, others proteins are: RP-A protein, which binds single-stranded DNA, Rad52, which can bind DNA ends and anneal complementary single-stranded DNA molecules, and a number of Rad51 paralogs (in human: Rad51-B, Rad51-C, Rad51-D, XRCC2, XRCC3). The process begins with the formation of the 3' single-stranded tails. The

3' ends are the substrate for the Rad51 monomers, which form a nucleoprotein filament. This filament joints the heteroduplex molecule formed between the damaged DNA and the undamaged template. In addition to Rad51, these steps require the coordinated action of the RP-A protein, Rad52 and Rad51 paralogs. The missing sequence in the heteroduplex is generated by DNA polymerase alpha. The continuity of the strands is established by a DNA ligase. The final step includes the separation of the intact duplex DNAs, in a process called 'resolution'. Recent studies have shown that in humans, other proteins called BRCA-1 and BRCA-2 are involved in the regulation of homologous recombination. It has been suggested that the BRCA proteins interact with Rad51 monomers to limit their association with double strand breaks (Zhang et al., 1998).

1.3.7e.2 Non-homologous end joining

Non-homologous end joining (NHEJ) is the more important double strand break repair mechanism in mammalian cells (Khanna and Jackson, 2001). The process starts with an association between the DNA ends and the protein kinase Ku. Subsequently the Ku protein recruits DNA dependent protein kinase. The ligase 4/XCC4 complex then joins the DNA ends. During the NHEJ process small deletions are generated. Given that majority of the mammalians genome is composed of non-coding regions, the probability that in normal situations the NHEJ process induces mutation in genes is low (Alberts et al., 2002e). However, if there are multiple break points NHEJ increases the occurrence of illegitimate recombination (Rothkamm et al., 2001).

1.3.7d Chromosome Instability (CIN)

The cell reproduces by a series of events that allow DNA replication and cell division in a process known as the cell cycle. In order to check the correct order of events that take place in the cell cycle, a complex cell-cycle control system has evolved (Alberts et al., 2002b). This system checks normal cell cycle progression by a series of stage-specific sensors known as checkpoints that are able to induce the arrest of the uncompleted stage until it is completed.

The two fundamental processes in the cell cycle are the duplication and the division of the chromosomes, which take place during the Synthesis (S) and Mitosis (M) phase respectively. To prevent the possibility that two daughter cells have non-identical genomes, there are two checkpoints known as DNA replication and DNA damage checkpoints before mitosis, and one known as spindle-attachment checkpoint during mitosis (Alberts et al., 2002b).

The DNA replication checkpoint stops the mitotic cycle until the total replication of the genomes is completed. The DNA damage checkpoint is present at two cell cycle stages; one in G1, which prevents entry in to S phase, and one in G2 preventing entry into mitosis (Alberts et al., 2002b). While the G2 checkpoint acts in the same manner as the DNA replication checkpoint, the damaged DNA activates a series of protein kinases that inactivate the Cdc25 phosphatase, blocking the activation of the cyclin B-cyclin dependent kinase 1 complex, known also as M-Cdk, which prevents entry into mitosis (Alberts et al., 2002b).

The G1 checkpoint acts by inhibiting the activation of the G1/S-Cdk and S-Cdk complexes. This inactivation is mediated by the regulatory protein p53. The damaged DNA activates protein kinase ATM that by the phosphorylation of p53 favours the dissociation of the ubiqitin ligase MDM2 from p53, thus decreasing p53 degradation.

Cells must pass the correct number of chromosomes to their progeny. The spindle attachment checkpoint prevents the altered segregation of the chromosomes during mitosis by monitoring the kinetochore status (Amon, 1999; Hoyt, 2001) . The kinetochore is a chromosome region that allows chromosomes to bind to the spindle's microtubules. The unattached chromosome binds to the mitotic checkpoint complex (MCC). This process is mediated by a ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C), along with a protein called Cdc20 (Amon, 1999; Hoyt, 2001).

Aneuploidy is the presence of abnormal chromosome numbers within cell (Rajagopalan and Lengauer, 2004). Generally the degree of chromosomal abnormality increases with the tumour progression being higher in malignant tumours. Aneuploidy is the outcome of diverse genetic and cellular alterations leading to unfaithful chromosome segregation during mitosis. Although it may be assumed that almost all steps during mitosis can, if altered, cause chromosome imbalance, mounting evidence indicates that the mitotic spindle checkpoint alteration is a crucial point for chromosomal instability (CIN) (Rajagopalan and Lengauer, 2004).

Chromosome instability (CIN) is also associated with structural alteration of chromosomes, which include, reciprocal and non-reciprocal translocations, amplifications, deletions and insertions (Gollin, 2005). Structural chromosome instability, resulting from DNA breaks and rearrangements, is due to alteration of cell cycle checkpoints, DNA damage response and telomere integrity (Gollin, 2005). Structural alterations may results in altered gene expression or produce fusion or chimeric proteins with dysregulated or new properties (Greaves and Wiemels, 2003).

Recent studies have shown that a large proportion of human tumours with chromosome instability have a high rate of loss of heterozygosity (Rajagopalan and Lengauer, 2004). Therefore it has been argued that chromosome instability could accelerate the rate of inactivation or activation of tumour suppressor genes or oncogenes respectively (Rajagopalan and Lengauer, 2004).

CIN-associated genes can be classified on the basis of the mutational events required to trigger instability (Michor et al., 2005). Class I CIN genes, such as MAD-2 (Mitotic Arrest Deficient) trigger CIN if one allele is mutated or lost. Class II CIN genes, such as hBUB-1 (human Budding Uninhibited by Benzimidazoles) trigger CIN if one allele is mutated in a dominant negative fashion. Both MAD-2 and hBUB-1 are required at the spindle assembly checkpoint (Amon, 1999; Hoyt, 2001). Class III CIN genes, such as BRCA1 and BRCA2 (Breast Cancer), trigger CIN if both alleles are mutated. BRCA genes are involved in DNA repair and recombination and checkpoint control of the cell cycle (Yarden et al., 2002).

1.4 Evolutionary Dynamics of Tumour Development

According to neo-Darwinism, evolution is a balance between inherited genetic changes and natural selection (Ridley, 2004b). Multicellular organisms develop from an initial diploid cell derived by the fusion of haploid germ cells in a process known as fertilization. This kind of development is called Weismannistic, after August Weismann who first expounded the distinction between somatic and germ cell lines (Ridley, 2004a). The separation between somatic and germ cells, which occurs early in development, focuses the action of natural selection on the germ cells, since only they transmit genetic information to future generations (Ridley, 2004a). The sources of genetic changes in germ cells are predominantly due to recombination between parental chromosomes and minimally to random mutations.

Numerous genes present in the germ line are selected because they restrain somatic conflict (Michod, 2002).

According to clonal evolution theory, cancer is the result of somatic mutations selected during tumour evolution (Nowell, 1976). It has been argued that tumour cells cannot acquire the mutations needed for tumour progression at a physiological mutation rate, but that the tumour cell must acquire an increased mutation rate (Cairns, 1998; Loeb and Loeb, 2000).

Given that cancer is the result of the accumulation of somatic mutations selected during tumour evolution, the processes of mutation and natural selection play an essential role in cancer biology. In order to induce cancer the mutations must affect a variety of genes that restrain somatic conflict (Frank and Nowak, 2004). These genes are known as cancer related genes and can be subdivided in three categories: Gatekeeper, Caretaker, and Landscaper (Michor et al., 2004).

Gatekeeper mutations increase the cellular proliferation rate by the alteration of oncogenes, tumour suppressor genes and apoptotic genes (Michor et al., 2004). Caretaker mutations increase genome instability by inactivating genes involved in maintaining genome integrity (Lengauer et al., 1998). Landscaper mutations increase tumour proliferation by affecting genes involved in regulating the external cellular microenvironment (Bissel and Radisky, 2001).

According to the multistage theory of cancer, tumours evolve by accumulating mutations not only in tumour initiating genes but also in genes involved in tumour progression (Michor et al., 2004). Although mutations in gatekeeper and landscaper genes induce a selective advantage because they increase the proliferation rate, the accumulation of mutations in caretaker genes are more problematic to explain (Breivik, 2005). Given that mutations of caretaker genes increase the mutation rate and that the majority of mutations are expected to be deleterious, natural selection

should limit the fixation of caretaker mutations. It has been argued that an elevated mutation rate could be advantageous because it allows the accumulation of mutations in gatekeeper and landscaper genes (Michor et al., 2004).

Theoretical analysis argues that in asexual organisms the mutated allele responsible for the increase in the mutation rate could be fixed in a population by hitchhiking with an associated allele that increases individual fitness. In sexual organisms the recombination process limits the hitchhiking mechanism (Sniegowsky et al., 2000).

Recent reports have suggested that the increase of the mutation rate in asexual organisms may be a mechanism of adaptation and that the mutation rate can increase in stressful situations, where more adaptation is required, and then decrease when the stress event is reduced (Cairns, 1998; Sniegowsky et al., 2000). Recent mathematical analysis shows that tissue organization and cell population size can influence the dynamics of tumour progression. In large populations natural selection favours the fixation of advantageous mutations, whereas in small populations, random genetic drift allows fixation of the deleterious and advantageous mutations at the same rate (Michor et al., 2003).

Tissues of multicellular organism are organized in compartments. The number of cells in each compartment is maintained constant by several mechanisms, and therefore the number of dead cells is replaced by new cells (Frank, 2003). In skin and intestinal epithelium the stem cells renew the compartment. In each division a stem cell generates one transit cell and a new stem cell. The transiting cell divides until it reaches the upper layer and later sloughs off. Therefore mutations occurring in the stem cells stay in the compartment and might accumulate, increasing the risk of cancer, while mutations occurring in the transit cell are eliminated from the compartment (Cairns, 1975).

These observations suggest that in rapidly renewing tissues organised into many small compartments, like skin and intestinal epithelium, where each compartment is renewed by few stem cells, there is little mixing of cell population between neighbour compartments, and mutations in caretaker genes are fixed by random genetic drift despite being disadvantageous (Michor et al., 2004). In contrast, in tissues with large compartments, where few stem cells produce large numbers of differentiated cells, such as bone marrow, or conditionally renewing tissues such as liver and kidney, natural selection favours the fixation of mutations affecting the gatekeeper and landscaper genes (Michor et al., 2004) . While mutations affecting oncogenes behave in a dominant way, because only one mutated allele can induce a tumour phenotype, mutations affecting tumour suppressor genes can be neutral if the normal allele compensates the mutant allele, disadvantageous if the mutant allele triggers apoptosis, and advantageous if the mutated allele is inactivated and the second allele is insufficient to balance the wild type allele (Michor et al., 2004). In small compartments the inactivation of the two alleles of a tumour suppressor gene, is unlikely, unless the mutation rate is increased by genetic instability (Nowak et al., 2005). Given that chromosome instability (CIN) increases the rate of loss of heterozygosity (LOH) it also increases the probability of inactivating two TSG alleles in a short time (Michor et al., 2004). Thus mutations in caretaker genes may occur at an early stage of tumorigenesis in tissues with small compartments (Michor et al., 2004).

1.5 Canine Transmissible Venereal Tumour

1.5.1 Geographical Distribution

Although no detailed epidemiological data are available about the prevalence of the CTVT in different countries, the tumour has been reported throughout the world. It has been recorded in France (Marchal et al., 1997), Spain (Mozos et al., 1996)

Poland (Wasecki, 1977), Australia (Locke et al., 1975), Russia (Osipov and Golubeva, 1976), Japan (Oshimura et al., 1973), Africa (Jackson, 1936) (Vermooten, 1987), Israel (Cohen, 1978), India (Das and Das, 2000), Turkey (Guvenc et al., 2002), Brazil (Saliba, 1958), United States of America (Hayes et al., 1983), Puerto Rico, Papua and New Guinea (Rust, 1949), and Bahamas (Higgins, 1966). In Italy CTVT is more prevalent in the southern regions (Ajello, 1936).

CTVT is rare in Denmark and United Kingdom, where the tumour has been only described in imported dogs. CTVT has never been reported in Sweden (Das and Das, 2000). Although the tumour has been described in many countries, CTVT is more prevalent in temperate areas with a higher prevalence in stray and feral dogs.

1.5.2 Transmission

Although the tumour is usually transmitted by coitus, it may be also transmitted by licking, sniffing or scratching tumour-affected areas (Feldman, 1929). Under natural conditions venereal transmission is highly efficient. Smith and Washbourn (1898) reported the transmission of the tumour to 12 bitches by one male dog. Ajello reported that in 590 cases, 400 were found in females (Ajello, 1980). It was hypothesised that extra-genital forms (skin) without genital involvement may be explained by the possibility of tumour cell transmission by an ectoparasite (Batamuzi and Bittegeko, 1991).

Although naturally occurring tumours have been described only in dogs, the tumour can be experimentally transmitted by inoculation in other species of the *Canidae* family such as: foxes (Sticker, 1906), wolves (Dungern, 1912), jackals (Samso, 1965), and coyotes (Cockrill and Beasley, 1979). The tumour cannot be experimentally transmitted to immunocompetent animals such as: mice, rats, hamster, cats, rabbits, chicken, monkeys, or mules (Smith and Washbourn, 1898;

Sticker, 1905; Ajello, 1939; Cockrill and Beasley, 1979). The tumour has been experimentally transmitted as a xenograft in nude mice (Holmes, 1981), X-irradiated mice (Stubbs and Furth, 1934), cortisone-treated hamsters (Samso, 1965) and SCID (Severe Combined Immuno Deficient) mice (Harmelin et al., 2001).

1.5.3 Etiology

Since the first description of the CTVT by Novinski in 1876 (Novinski, 1876), numerous experiments have been carried out to determine the etiology of the tumour. Although CTVT was generally described as a tumour, Bashford, Murray, Cramer at the newly established Imperial Cancer Research Fund, suggested that CTVT was not a tumour but a connective tissue reaction to a virus (Bashford et al., 1905). Also, the high reproducibility of the tumour in experimentally transplanted dogs was a strong argument for a viral aetiology of CTVT (Gross, 1983). While experimental transmission of the tumour by inoculation of intact and living tumour cells was highly reproducible (Smith and Washbourn, 1898; Sticker, 1904; Stubbs and Furth, 1934; Ajello, 1936; Karlson and Mann, 1952; Smith, 1976), in spite of numerous efforts, transmission of the tumour by inoculation of cell free filtrates was reported only by Thierry (1950), Ajello (1960) and Dozza and Torlone, (1960). Experimental transplantation using labelled tumour cells indicated that the tumour developed from the transplanted tumour cell (Kudo et al., 1974). Several cytogenetic studies showed that tumours from different countries are characterized by constant chromosome rearrangements (Makino, 1963; Weber et al., 1965; Murray et al., 1969).

To date there is no evidence indicating a specific pathogen that induces the tumour, and the only way to transmit the tumour remains the inoculation of living tumour cells.

1.5.4 Anatomo-Pathological and Clinical Aspects

The tumour is located mainly in the external genital organs. In the male the tumour is usually located in the glans penis and occasionally in other parts of the penis and prepuce. In the female it is usually located in the posterior part of the vagina in proximity of the vestibule and occasionally may protrude from the vulva (Cohen, 1985). The tumour on the external genitalia appears as a cauliflower-like, pedunculated or multilobulated mass that can reach up to 15 cm of diameter (Ajello, 1980). In some cases the tumour mass may infiltrate the surrounding region: mammary gland, scrotum, rectum and peri-anal and perivulvar regions (Ajello, 1980). Metastases have been frequently described in immunosuppressed dogs and puppies and are mainly located in the inguinal or iliac lymph nodes, and occasionally in spleen, liver, brain, lungs, oro-nasal cavity, and eyes (Prier and Johnson, 1964; Ajello, 1980; Padovan et al., 1987; Placke et al., 1987; Das and Das, 2000; Pereira et al., 2000). Extra-genital localizations have been described in external organs like eyes, mouth, skin and nose, also without genital involvement and in virgin dogs (Feldman, 1929; Jackson, 1936; Ajello, 1939; Nayak and Samaddar, 1988; Batamuzi and Bittegeko, 1991; Zanghi et al., 1996).

In natural and experimental conditions the tumour starts to develop from the sub-mucosal layer and grows toward the mucosal layer causing distension and subsequently ulceration of the mucosal surface, assuming the clinical appearances described below (Ajello, 1980; Cohen, 1985). Tumour growth beneath the submucosal tissue is slow, but in some cases may be more extended than the superficial one, specially when the tumour affects subcutaneous tissues, and in these cases the tumour grows rapidly under the skin and slowly infiltrates the skin tissue causing ulceration (Ajello, 1980). Usually clinical signs include serous or sanguineous discharge from the genital areas. Several CTVT cases associated with mechanical obstruction of the urethra and uterus have been reported (Rust, 1949; Batamuzi and Kristensen, 1996). From clinical observations it has been suggested

that the tumour becomes clinically evident in a period ranging from 14 days to 3 months after sexual intercourse (Ajello, 1939; Ajello, 1980). CTVT is usually described in adult (2-5 years old) and sexually active dogs (Rogers et al., 1998; Das and Das, 2000). Although both sexes are susceptible, in some studies the tumour was found to be more prevalent in females (Ajello, 1980). In endemic areas the tumour is more frequently observed in stray or feral dogs. However, naturally occurring CTVT has been described in many dog breeds.

Although experimentally induced tumours regress spontaneously after 3-6 month, the frequency and the period of regression has not been accurately determined in naturally occurring CTVT (Higgins, 1966; Ajello, 1980; Yang, 1987; Yang, 1988). Normally in healthy animals tumours regress after 3-9 months, but in some cases, particularly when the animals are aged or unhealthy, the tumours do not regress (Das and Das, 2000). CTVT has occasionally been reported in association with Leishmaniasis (Albanese et al., 2002; Catone et al., 2003) and recently the presence of the *Leishmania infantum* within the CTVT cells has been described (Catone et al., 2003). In these cases the immune suppression caused by the Leishmania parasite appears to favours the diffusion of the tumour to external and internal organs.

1.5.5 Histological and Cytological Features

Despite extensive and detailed studies, the histogenesis of the tumour is still debated. Early histological observations described the tumour as myxosarcoma (Novinski, 1876), carcinoma (Wehr, 1888), lymphosarcoma (Sticker, 1905), round cell sarcoma (Smith and Washbourn, 1898), infective granuloma (Bashford et al., 1905), tumour of neuro-ectodermal origin (Jackson, 1944), and histiocytoma (Mulligan, 1949). The tumour has been defined as a round cell tumour of reticuloendothelial origin (Cockrill and Beasley, 1975) but the term, reticulo-endothelial cell, has fallen out of fashion. Similarly the term, histiocyte, is less

frequently used today, but it denotes non-lymphocytic, phagocytic cells of haematropoietic origin that reside in solid tissues. Immunophenotypic studies showed that the tumour cells are alpha -1- antitrypsin, lysozyme and ACM1 positive, suggesting a histiocytic origin of CTVT (Mozos et al., 1996; Marchal et al., 1997; Perez et al., 1998; Mukaratirwa and Gruys, 2003). The presence of *Leishmania infantum* within the CTVT cells, supports an histiocytic origin of CTVT (Catone et al., 2003) because this parasite invades phagocytic cells such as macrophages and dendritic cells. In the haematoxylin-eosin stained sections the tumour cells appear round with a diameter ranging from 15 to 30μm, with a round-oval nuclei containing coarsely aggregated chromatin and usually a prominent nucleolus (Cohen, 1985). The cytoplasm is abundant and is distinct in areas where the tumour cells are not densely packed; it is lightly basophilic and usually contains vacuoles. Histologically the tumour cells are packed and arranged in diffuse clusters intercalated by a delicate stroma (Ajello, 1980).

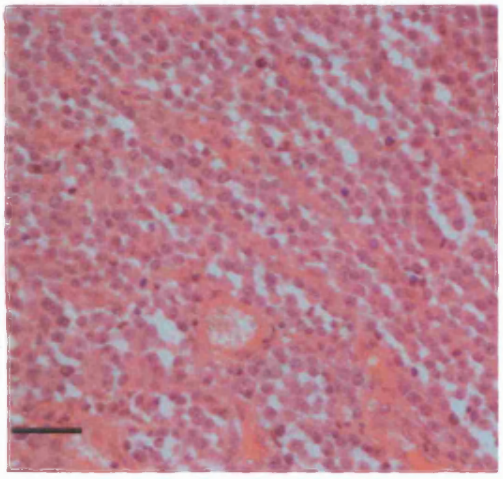


Fig 1. Histopathology of CTVT. Microscopic section of tumour in progressive stage. Hematoxylin-esoin stain. Bar = 60 μm

Ultrastructural studies showed that tumour cells contain an extensive network of granular endoplasmic reticulum, a prominent Golgi apparatus, and numerous ribosomes (Cockrill and Beasley, 1975; Kennedy et al., 1977; Otomo et al., 1981). A cytoplasmic lamellar array has also been described (Cockrill and Beasley, 1975).

Several studies showed that tumour cells in culture undergo a morphological transformation from round cells to fibroblast-like cells (Adams et al., 1968; Kennedy et al., 1977). Several reports have shown the presence of crystalline virus-like structures in the tumour cells (Ajello and Gimbo, 1965; Lombard and Cabanie, 1967; Battistacci and Morriconi, 1974)

1.5.6 Cytogenetic Features

The normal diploid chromosome number in dogs, wolves, jackals and coyotes is 78. The autosomic chromosomes are acrocentric, whereas the X chromosome is large and submetacentric, and the Y chromosome is small and metacentric (Breen et al., 2001)

In the 1960s Takayama and Makino analysed the CTVT karyotype from different locations in Japan and showed that this is characterized by 59 chromosomes, of which 17 were metacentric and 42 acrocentric (Takayama and Makino, 1961). Several observations showed that the karyotype of tumours from different countries are characterized by 57-59 chromosomes, with 15-17 metacentric and 42-44 acrocentric chromosomes (Makino, 1963; Weber et al., 1965; Barski and Cornefert-Jensen, 1966; Kakpakova ES et al., 1968; Murray et al., 1969; Idowu, 1977; Richardson et al., 1987) (Figure 1.2).

Cytogenetic studies performed in tumour cells grown in vitro revealed the same chromosomal rearrangements (Adams and Slaughter, 1981). It has been also reported that the number of the chromosome arms and the DNA content in the tumour are approximately the same as in the normal diploid karyotype, and thus it was proposed that the metacentric chromosomes observed in CTVT arose from centric fusion (Weber et al., 1965; Barski and Cornefert-Jensen, 1966; Wright et al., 1970). From these cytogenetic studies it was postulated that all CTVT tumours

worldwide might have been derived from a single tumour clone expansion through tumour transplantation.

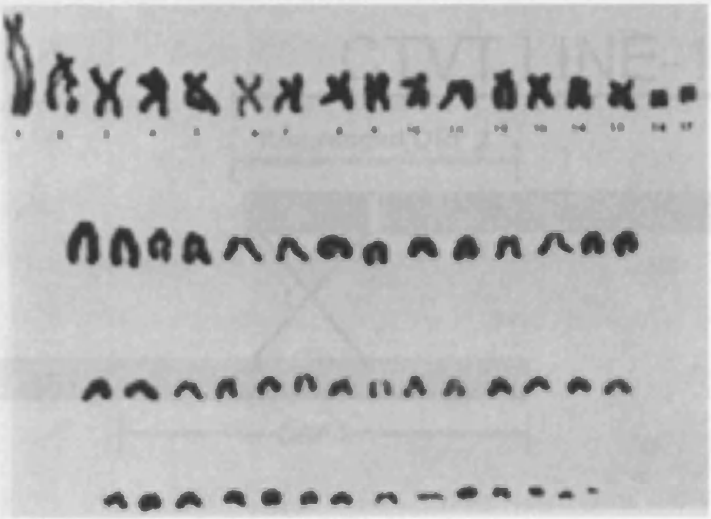


Figure 1.2. CTVT karyotype. Reproduced with permission from Max Murray (Murray et al, 1969)

1.5.7 Molecular Markers

In a study on the involvement of oncogenes in the genesis of the tumour, a Long Interspersed Nuclear Element (LINE-1) was found to be inserted 5 Kb upstream to the first coding exon of the c-*myc* gene (Katzir et al., 1985). Additional studies reported the presence of the specific rearranged LINE/c-*myc* in tumours from different countries (Katzir et al., 1987; Amariglio et al., 1991). More recently it was reported that in two tumours from Japan, the inserted LINE-1 was 64 bp shorter than the LINE-1 previously described (Choi et al., 1999) and that that the total length of the inserted LINE-1 is not constant (Liao et al., 2003). The inserted LINE element is homologous to the canine LINE-1 ORF2, and contains a 416 bp region homologous to the complementary strand of the LINE-1 ORF 2, followed by a 5-bp deletion and 962-bp sequences homologous to the 3' region of the LINE-1 ORF 2 (Choi et al.,

1999) (Fig 1.3). The involvement of the truncated LINE-1 element on deregulation of c-*myc* transcription in the CTVT is not clear, although c-*myc* expression is high in the tumour (Katzir et al., 1987).

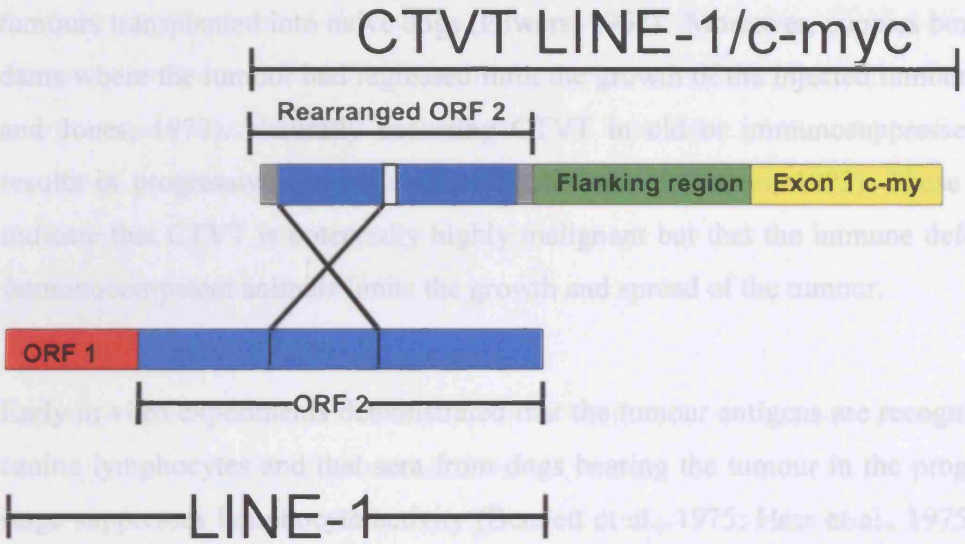


Figure 1.3. Diagram of LINE-1/c-myc rearrangement in CTVT. Gray rectangles represent the 10bp flanking direct repeats, white rectangle indicates 5bp deletion.

1.5.8 Immune Response to CTVT

In 1906 Sticker performed a detailed immunological study on CTVT and demonstrated that spontaneous regression of the tumour is followed by immunity against subsequent transplantation (Sticker, 1906). Although natural cases of CTVT have been reported only in dogs, the tumour has been experimentally transplanted into immunocompetent wolves, coyotes, foxes, and jackals and into immunosuppressed mice. Both natural and experimentally transplanted tumour exhibits two stages; in the first stage the tumour grows progressively increasing its size from a minute nodule to a pedunculated mass reaching up to a 15 cm of

diameter (Yang, 1988; Perez et al., 1998). This stage is usually followed by a regression stage, where the tumour decreases in size and assumes a friable consistency (Yang and Jones, 1973; Perez et al., 1998). Immunological studies have shown that the passive transfer of post-regressive sera inhibited the development of tumours transplanted into naive dogs (Powers, 1968). Moreover, puppies born from dams where the tumour had regressed limit the growth of the injected tumour (Yang and Jones, 1973). Naturally occurring CTVT in old or immunosuppressed dogs results in progressive growth and metastatic spread (Cohen, 1973). These results indicate that CTVT is potentially highly malignant but that the immune defence in immunocompetent animals limits the growth and spread of the tumour.

Early in vitro experiments demonstrated that the tumour antigens are recognized by canine lymphocytes and that sera from dogs bearing the tumour in the progressive stage suppresses lymphocyte activity (Bennett et al., 1975; Hess et al., 1975). The immunosuppressive activity of progressive tumours has been also supported by plasmapheresis experiments (Zander et al., 1980). Several studies have reported the presence of tumour specific antibodies in sera of dogs bearing tumour in progressive and regressive stages, and that tumour-specific antibodies could be detected 40 days after tumour transplantation (Dozza and Torlone, 1960a; Cohen, 1972). In spite of extensive efforts to identify the tumour antigens that drive the antibody response their nature remains unknown.

Immunological and histochemical studies have shown that during the progressive stage tumour cells do not express the Dog Leukocyte Antigens (DLA) class I and II, whereas these antigens are expressed during the regression stage (Cohen, 1974; Yang et al., 1987; Perez et al., 1998). It has been also reported that progressive tumour cells do not express β2-microglobulin (Cohen et al., 1984). Transplantation experiments suggested that the clinical course of the tumour is influenced by the diversity between tumour and host MHC (DLA) antigens (Epstein and Bennett, 1974).

Histological studies demonstrated that tumours in the regressive stage are much more infiltrated by lymphocytes and macrophages compared to tumours in progressive growth (Perez et al., 1998; Barber and Yang, 1999; Hsiao et al., 2002). A recent study reported that during progressive growth, the CTVT cells secret tumour growth factor beta (TGF-β1) and that during regression, tumour-infiltrating lymphocytes secrete an elevated amount of interleukin 6, so antagonizing the suppressive affect of the TGFβ1 (Hsiao et al., 2004).

1.5.9 Diagnosis

Although the CTVT has distinct clinical and anatomo-pathological features, cytological and histological examination is generally the most effective diagnostic method (Mozos et al., 1996; Marchal et al., 1997). Given the morphological similarities between CTVT and cutaneous histiocytoma, the diagnosis of extra-genital CTVT with conventional stained tissue sections may be difficult. In such cases epidemiological and clinical records may help the diagnosis. Although cytogenetic analysis may be a reliable diagnostic method (Makino, 1963; Murray et al., 1969; Osipov and Golubeva, 1976), it is not commonly used, but more recently the LINE-1/c-*myc* insertion has been used (Liao et al., 2003).

1.5.10 Therapy

Although generally CTVT regresses spontaneously in healthy individuals, several therapeutic approaches have been used to treat the tumour. Surgical excision of the tumor is practicable in the localized forms without severe infiltration, however tumour recurrence after surgical removal without chemotherapy has been reported (Karlson and Mann, 1952; Prier and Johnson, 1964; Das and Das, 2000). Several reports have shown that the CTVT is sensitive to radiotherapy (Osipov and

Golubeva, 1976; Ajello, 1980). The application of 1000 rad in 2 or 3 treatments is generally sufficient to induce the complete regression (Thrall, 1982). Although radiotherapy is highly effective, it requires chemical sedation of the animal and expensive equipment. Chemotherapeutic agents have been used to treat the tumour (Osipov and Golubeva, 1976; Ajello, 1980; Epstein and Sarpel, 1980; Das and Das, 2000). Vincristine sulphate or vinblastine seem to be most effective and with less collateral side effects than other chemotherapeutic agents. Generally six intravenous injections of Vincristine (0.025mg/Kg body weight) at weekly intervals are sufficient to induce complete regression (Brown et al., 1982; Singh et al., 1996).

1.6 The Canine Major Histocompatibility System

The canine Major Histocompatibility System (MHC) is also known as Dog Leucocyte Antigen (DLA). It contains clusters of genes encoding proteins that are involved in the establishment and regulation of the immune response. The DLA region has been mapped to chromosome 12q telomeric by fluorescence in situ hybridization (FISH) (Dutra et al., 1996). This region shows a high level of conserved synteny among the MHC of human, mouse and cat species (Debenham et al., 2005).

From immunological and structural studies the MHC genes have been subdivided in three classes: Class I, Class II and Class III. While Class I and Class II genes are involved in antigen presentation the Class III region contains genes coding for proteins involved in the coagulation process and in the immune regulation . Some of the Class I and Class II genes show a high degree polymorphism (Wagner et al., 1999).

Based on the transcription level and polymorphism the Class I genes are divided in to classical (more polymorphic and high transcription) and non-classical (almost

monomorphic and low transcription) genes (Burnett et al., 1997). The DLA class I region contains several non-classical genes and pseudogenes, but only one classical gene, DLA-88, is polymorphic among unrelated dogs (Burnett et al., 1997) (Graumann et al., 1998). Although the majority of the DLA genes are located on chromosome 12, the non-classical gene DLA-79 has been mapped on chromosome 18, but its function is unknown (Burnett and Geraghty, 1995).

The DLA class II region like the MHC Class II region in other species is divided into sub-regions BTNL2-DOB, DOB-DOA, DOA-COL11A2, COL11A2-DAXX (Debenham et al., 2005). From the immunological point of view the most important region is the BTNL2 region. This sub-region contains the DR and DQ loci. The dog has two functional DR genes (DLA-DRA1 and DLA-DRB1) and two functional DQ genes (DLA-DQA1 and DLA-DQB1) (Kennedy et al., 2000; Debenham et al., 2005).

Class I MHC proteins consist of a large protein with a transmembrane region (alpha chain) and an associated small extracellular protein (β2- microglobulin) which is not encoded by MHC genes (Wagner et al., 2001). The alpha chain is folded to form three extracellular domains (1, 2, 3). While the alpha-3 domain is constant and is associated with the β2 microglobulin, the alpha 1 and 2 domains are polymorphic and together form the groove that binds peptide antigens to present them to cytotoxic cells (Roitt et al., 1988). The Class II proteins are heterodimers, composed of two transmembrane chains, alpha and beta. Both chains consist of one constant (C) domain and one variable (V) domain. The two variable domains interact to form the peptide-binding groove (Roitt et al., 1988). The role of the MHC Class I and Class II proteins in binding and presenting the antigens for immune recognition explains the extensive polymorphism present in these MHC genes.

Several studies have shown that the genetic variability of the MHC genes is under the influence of balancing selection, which maintains the variability and

heterozygosity level beyond neutral expectations (Hedrick, 1998; Richman, 2000; Hedrick et al., 2002; Garrigan and Hedrick, 2003). Transplantation and immune-genetic studies have shown large variability in several species of the *Canidae* family (Wagner et al., 2002). Recently it has been shown that the level of variation of the DLA class II haplotypes in dogs is higher between breeds but is minimal within a breed (Kennedy et al., 2002). Although the level of homozygosity varies between DLA class II genes, the level of homozygosity tends to be higher in rare breeds than the common breeds, perhaps because they derive from a smaller number of founder animals (Kennedy et al., 2002). Given the high degree of genetic polymorphism in canine DLA-88 (Class I) and in DLA-DRA, DLA-DRB, DLA-DQA and DLA-DQB (Class II) genes, DLA markers are useful for the genetic analysis of CTVT.

1.7 Microsatellites

Eukaryotic genomes are full of repeated DNA sequences. These tandemly repeated sequences are classified by the length of the core unit and the length of the contiguous repeat units, into three main divisions: (a) Satellites, which are often associated with centromeres, where a single unit is repeated to form an array up to 5 MB long; (b) Minisatellites, also known as variable number of tandem repeats (VNTRs) in which a unit of 10-100 bp is repeated to give an array of 0.5-3 kb size; (c) Microsatellites also known as short tandem repeats (STRs) are sequences containing a core unit of 2-8 bp, tandemly repeated and are distributed throughout the genome (Armour et al., 2001).

The discovery that repeated DNA sequences show variations in the number of tandem repeats known as variable number of tandem repeats (VNTRs) (Jeffreys et al., 1985a), which differ from individual to individual, led to the development of the DNA fingerprint technique (Jeffreys et al., 1985b). Given the wide distribution of microsatellites throughout the genome and that the number of the repeats can be

highly variable among individuals, microsatellites are widely used in evolutionary and genetic studies and in forensic science. Although several types of mutations may affect the microsatellites, the elevated mutation rate is mainly caused by changes in the number of repeats, a situation referred as microsatellite instability (MSI) (Eisen, 2001). The mechanism of the mutation process is still debated, and two models have been proposed. One model suggests that microsatellite instability is due to the recombination by unequal crossing over (UCO) between homologous chromosomes or by gene conversion (Smith, 1976). The alternative model proposes that MSI is caused by slip-strand mispairing (SSM) errors occurring in the DNA replication process. Several studies support the SSM model as the major mechanism involved in the microsatellite instability (Sia et al., 1997). It has been shown that the nature of the microsatellite itself plays an important role on the SSM mechanism. The mutation rate has been found to increase when the core unit and the number of repeated units increase (Wierdl et al., 1997; Bachtrog et al., 2000). The nature of microsatellites not only influences the mutational mechanism but also repair activity. Several studies suggest that long microsatellites form a loop when they are misaligned and that the mismatch repair machinery repairs the short size loops better than the long ones (Kroutil et al., 1996). The mutation mechanism and the repair process can be influenced by several factors that include the local DNA sequences (CG content), genome position (chromosome position), methylation state and level of transcription (Eisen, 2001).

In order to optimize the information provided by microsatellites for genetic and phylogenetic applications, two principal evolutionary dynamic models have been postulated: The infinite allele model (IAM) and the step wise mutation model (SMM). According to the IAM model, microsatellites may mutate by gaining or losing any number of tandem repeats (Kimura and Crow, 1964). In contrast, the SSM model postulates that the mutation involves the loss or gain of a single repeat unit (Kimura and Otha, 1978). Although the gain or loss of the repeat may have the same probability, genetic analysis shown that gain occurs more frequently than loss (Amos and Rubinstzein, 1996; Primmer et al., 1996). The propensity of

microsatellites to gain repeats would theoretically promote an infinite expansion of the microsatellites. This is in contrast with the fact that genomes rarely have long repeats compared to short repeats. This suggests that there must be a size constraints which restrict the repeat expansion (Amos, 2001). It has been estimated that the mutation rate (changes in repeat number) of microsatellites is higher than the mutation rate of point mutations (Ellegren, 2000). Estimates from pedigree analysis in humans suggest a rate around 10^{-3} mutations per locus per generation (Weber and Wong, 1993), and 10^{-3} to 10^{-4} in mice (Dallas, 1992) and 10^{-2} to 10^{-4} in dogs (from Elaine Ostrander, personal communications).

Given their high variability and their wide genome distribution, microsatellites are widely used for gene mapping, forensic analysis, conservation biology, and phylogenetic studies in several species. Thank to the efforts in developing the dog genome map, to date 1,589 canine microsatellite markers have been characterized (Ostrander and Wayne, 2005). The canine microsatellite markers have become the tool of choice in several applications. In conservation genetics microsatellites have been used to analyze the divergence between populations, hybridization and introgression between canine species, social structure and inbreeding (Gottelli et al., 1994; Vila et al., 1999b; Lucchini et al., 2002; Vila et al., 2003).

Since the domestication of dogs from wolves, dogs have been selected for a specific body size, conformation, pelage, temperament and behaviour, thus undergoing a high diversification into different breeds. More than 400 breeds have been recognized throughout the world, displaying a very high phenotypic diversity. To maintain the physical and behavioral features to a specific breed, domestic dogs have undergone inbreeding and isolation, so contributing to the propagation and high prevalence of the breed specific genetic traits (Wayne, 2001). To exploit this breed related genetic information, microsatellites are a crucial tool to map genes important in diverse physiological and pathological situations.

According to the American Kennel Club (AKC) there are seven major groups of dogs breeds, classified as follows: Herding, Sporting, Hound, Working, Terrier, Toy and non-Sporting. Recently canine microsatellites have been used to determine the genetic relationship between 85 breeds with a world-wide distribution (Parker et al., 2004). Results indicate that 99% of 414 individual dogs analyzed can be assigned to an original breed. The 85 breeds can be separated by clustering methods in to 4 clusters, each containing breeds with similar behaviour, morphology and geographical origin. Phylogenetic analysis separates these breeds into two clusters according to an ancient or modern origin.

From the foregoing discussion, there is a strong case for using microsatellite DNA to analyze CTVT. First, microsatellites can be used to determine whether the tumour differs from the host; second, if that is the case, microsatellites can be used to indicate the breed of origin of CTVT.

1.8 Mitochondrial DNA

Mitochondrial DNA (mt-DNA) is a circular molecule of 16,500 base pairs and it is located within the mitochondrion. There are about 100 mitochondria per cell and about 10 copies of mt-DNA per mitochondria. The number of mt-DNA copies within the cell is variable and depends on the cell type. In mature sperm there are about 50-80 mitochondria, while the mature oocyte, a large cell, contains more than 100,000 mitochondria (Chinnery et al., 2000).

During fertilization the mitochondria present in the sperm do not penetrate into the oocyte, and thus the embryo contains only the maternal mitochondria. This type of inheritance is called non-Mendelian or cytoplasmic inheritance (Alberts et al., 2002i). Given that the paternal mitochondria are not inherited, recombination between maternal and paternal mt-DNA does not take place, and therefore the only

source of variation is due to mutation (Chinnery et al., 2000). Although mt-DNA has generally been assumed to be maternally inherited, recent studies have suggested that a small fraction of mt-DNA may sometimes be paternally inherited (Roze et al., 2005).

The majority of the mt-DNA contains genes that encode ribosomal RNAs, tRNAs and several subunits of the respiratory chain. However, there is a small non-coding region called the Control Region (CR) or Displacement-loop (D-loop) which is involved in the regulation of the replication and transcription (Alberts et al., 2002i).

In contrast to the nuclear genome, mitochondrial DNA molecules lack histones, and therefore mt-DNA is more susceptible mutation than nuclear DNA. It has been estimated that the mt-DNA mutation rates is 100-200 fold higher than nuclear DNA (Taylor and Turnbull, 2005).

The presence of reactive oxygen species (ROS) produced by the oxidative phosphorylation process and the attachment of the D-loop region to the mt-membrane, makes the D-loop region highly mutable (Mambo et al., 2003). A further factor that contributes to the high mt-DNA mutation rate is the absence of an efficient DNA repair system, due to the fact that the mitochondrial DNA polymerase exhibits a low proofreading activity (Alberts et al., 2002i). Given the high mutability of the D-loop region, the presence of different mt-DNA haplotypes (heteroplasmy) has been reported within the individual or cell or single mitochondria (Shin et al., 2004a; Shin et al., 2004b).

Recent studies have shown that the accumulation of somatic mutations in the human mt-DNA control region in normal individuals is age-dependent (Michikawa et al., 1999; Elson et al., 2001; Trifunovic et al., 2004). However it has been suggested that expansion of preexisting mutants rather than de novo mutation in old age may be responsible for some aging-specific mutants (Shin et al., 2004b). The

accumulation of somatic mutation in mt-DNA has been also described in several human and animal tumours (Van Tuyle et al., 1996; Bianchi et al., 2001; Chen et al., 2004).

Several studies have described the presence of clonally expanded somatic mt-DNA mutations in normal and malignant tissues (Fliss et al., 2000; Shin et al., 2004b). Although the mechanism driving the mutated mt-DNA copies to homoplasmy is still unknown, these observations suggest a replicative advantage for mutated mt-DNA molecules within the mitochondria, and consequently a growth advantage for a cell containing certain mt-DNA mutations (Fliss et al., 2000; Bianchi et al., 2001). In addition, it is possible that some mt-DNA mutation may have a tumorigenic property (Petros et al., 2005). However, it has been argued that the observed homoplasmy might possibly have arisen entirely by genetic drift in tumor progenitor cells, without any physiological or pathological advantage (Coller et al., 2001).

The domestic dog (*Canis familiaris*) mitochondrial DNA has been sequenced (Kim et al., 1998), and the analysis of the mt-DNA control region variability has been used in forensic and phylogenetic studies (Wayne, 1993; Savolainen et al., 1997). Given the high degree of variation present in the control region and the high copy number within the cell, mt-DNA is a useful material for genetic investigation. It has been found that mt-DNA exhibits intra- and inter- breed variation (Okumura et al., 1996; Tsuda et al., 1997; Savolainen and Lundeberg, 1999). Therefore the mt-DNA may not be used to assign individual dogs to the original breed. A recent survey showed that the exclusion capacity of the mt-DNA is around 0.929 (Angleby and Savolainen, 2005).

The analysis of the mt-DNA control region in several species belonging to the *Canidae* family has shown that domestic dogs originate from at least four female wolf lines, identified as four different clades (Vila et al., 1997; Savolainen et al., 2002). The analysis of the mt-DNA control region in archeological dog specimens

from the Americas dated before European colonization has shown that the native America dog breeds come from the Old World dogs, thus refuting the New World domestication hypothesis (Leonard et al., 2002). A recent phylogenetic analysis of the mt-DNA control region of 654 dogs distributed worldwide has shown that the greatest genetic variation is present in East Asian dog populations, which suggests an East Asiatic domestication event, and that present day dogs diverged from the wolf lineage approximately 15,000 years ago (Savolainen et al., 2002).

The utility of mt-DNA for phylogenetic analysis among dogs means that this non-nuclear genetic marker can also be exploited to determine the evolution of CTVT.

1.9 Aim of Thesis Project

At the time that I began my thesis studies in 2001, there was strong circumstantial evidence, from karyotype analysis and from the common LINE-1/c-*myc* insertion that CTVT may represent a sexually transmissible cancer cell rather than a tumour caused by a sexually transmitted oncogenic virus. However, proof of this concept was still lacking, and no investigation had previously obtained and analyzed normal and tumour tissues from a series of dogs that had naturally acquired CTVT. I therefore set about collecting such matched tumour and normal samples, as well as archival paraffin-embedded tumour biopsies from five continents. I sought to determine whether the tumour was distinct from its host, and whether it represents one or more distinct clones by using a variety of polymorphic genetic markers in the canine genome. Having shown that CTVT indeed represents a single clone of cells, I have sought to determine the breed of origin of the tumour, and to estimate its approximate age.

CHAPTER 2

Material and Methods

2.1 Specimens

Matched tumour and blood samples were obtained from 15 unrelated stray dogs of different locations, breeds, age and sex as specified in Table 2. Upon clinical examination ten dogs showed primary external genital lesions (penis and vagina) characteristic of CTVT, and all dogs were in good general condition, whereas one dog showing multiple neoplastic lesions localized in the penis, skin and eyes, and was in poor general health. None of the dogs had received chemotherapeutic or immune stimulating drugs before specimen collection. Tumour tissues were obtained by surgical excision of primary and metastatic neoplasms. From 2 to 5 ml of peripheral blood were also collected. Tumour and normal (blood) tissues from Sicily were brought to the UK with permission of DEFRA and were tested for the absence of rabies by RT-PCR at the Veterinary Laboratory Agency (Weybridge) prior to use. DNA was extracted from tumours and blood samples of Indian (Kolkata) and Kenyan (Nairobi) specimens on site.

Thirty paraffin embedded tumour samples were collected from different geographical locations as specified in Table 2.

Table 2.1 Dogs providing fresh tumour and blood samples

Sample	Date	Breed	Sex	Age (yrs)	Tumour location	Country
A	2001	Mixed	Male	2	penis	Italy (Catania)
B	2001	Mixed	Male	1	penis	Italy (Catania)
C	2001	mixed	Male	10	penis eye skin lymph nodes	Italy (catania)
D	2001	Mixed	Male	2	penis	Italy (Catania)

E	2001	Mixed	female	5	vagina	Italy (Catania)
F	2003	Mixed	Male	3	penis	India (Kolkata)
G	2003	Mixed	Male	2	penis	India (Kolkata
H	2003	Mixed	female	2	vagina	India (Kolkata
I	2003	Mixed	female	3	vagina	India (Kolkata
L	2003	Mixed	Male	nd[†]	penis	Kenya (Nairobi)
M	2003	Mixed	Male	nd	penis	Kenya (Nairobi)
N	2005	Mixed	female		vagina	Italy (Messina)
O	2005	Mixed	famle		vagina	Italy (Messina)
P	2005	Mixed	female		vagina	Italy (Messina)
Q	2005	Mixed	female		vagina	Italy (Messina)

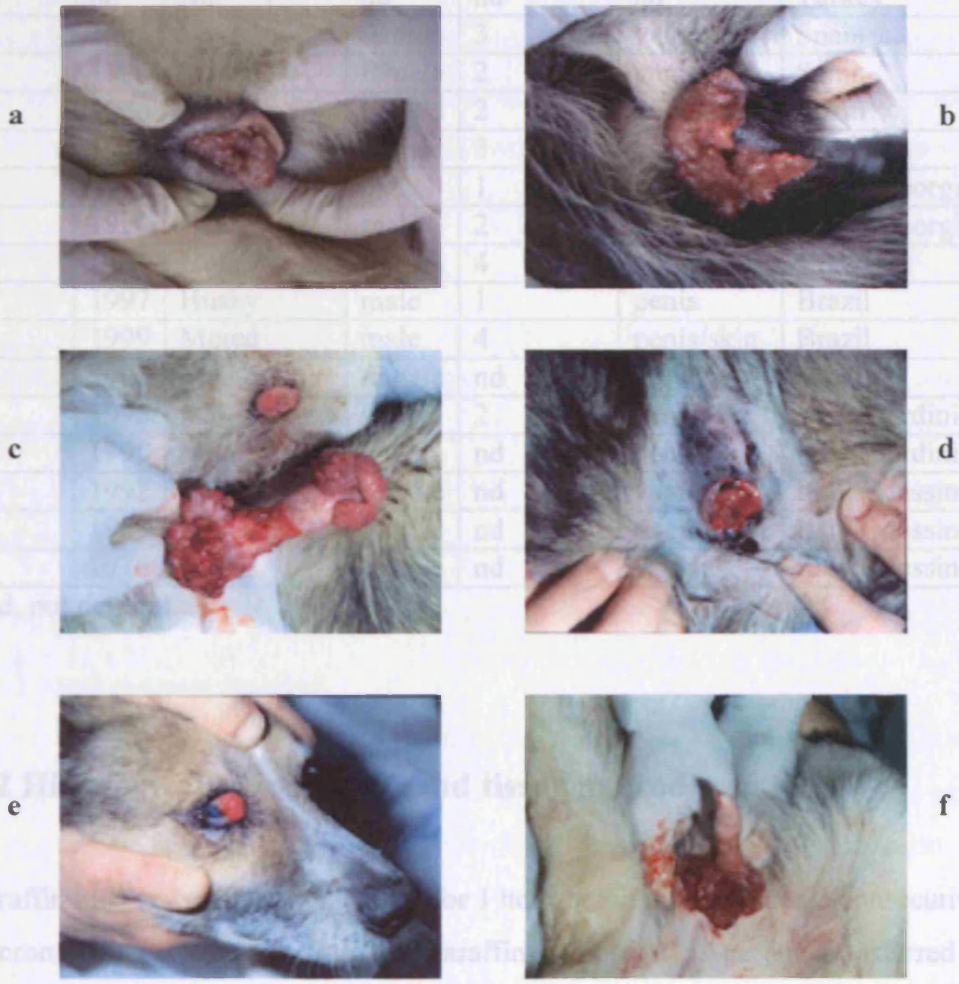


Fig 2.1 Canine Transmissible Venereal Tumour macroscopic appearance. **a, b**: CTVT in female dogs E and N; **c-e**: CTVT genital and extragenital (penis, skin and eye) in male dog C;**f**: CTVT on the glans penis of dog D.

Table 2.2 Paraffin embedded samples used for micro-dissection of tumours.

Sample	Date	Breed	Sex	Age (yrs)	Tumour location	Country
	nd†	Nd	nd	nd	nd	Turkey
3	nd	Nd	nd	nd	nd	Turkey
4	nd	Nd	nd	nd	nd	Turkey
5	nd	Nd	nd	nd	nd	Turkey
6	nd	Nd	nd	nd	nd	Turkey
8	nd	Nd	nd	nd	nd	Turkey
9	nd	Nd	nd	nd	nd	Turkey
11	nd	Nd	nd	nd	nd	Turkey
12	nd	Nd	nd	nd	nd	Turkey
14	1985	Mixed	female	3	vagina	Spain
17	1985	Mixed	female	2	vagina	Spain
18	1986	Mixed	female	2	vagina	Spain
19	1986	Mixed	female	3	vagina	Spain
20	1999	Mixed	male	1	penis	USA (Georgia)
21	1999	Beagle	male	2	penis	USA (Georgia)
25	1999	Husky	female	4	vulva	Brazil
27	1997	Husky	male	1	penis	Brazil
29	1999	Mixed	male	4	penis/skin	Brazil
30	1995	Mixed	female	nd	cervix	Brazil
32	1995	maremmano	male	2	penis	Italy (Sardinia)
33	1995	Mixed	male	nd	penis	Italy (Sardinia)
92	1992	Mixed	female	nd	vagina	Italy (Messina)
83	1983	Mixed	female	nd	vagina	Italy (Messina)
76	1976	Mixed	female	nd	vagina	Italy (Messina)

†nd, not determined

2.2 Histological examination and tissue microdissection

Paraffin blocks were placed at -20^{0}C for 1 hour before cutting, then 3 consecutive 5-micron sections were cut from the paraffin embedded tissue and transferred to a clean floating bath heated at 42^{0}C. The sections were then transferred to positively charges slides. After incubation for 30 minutes at 60 ^{0}C, sections were deparaffinised, submerged in to a glass tray containing xylene for 5 minutes, and transferred in a new tray containing clean xylene for further 5 minutes. After

deparaffinisation the sections were treated for 5 minutes with ethanol at 100%, 95% and 80% respectively and then washed with deionised water for 5 minutes. Middle sections were stained using standard haematoxylin-eosin staining for histological examination and the others were stained with methyl green for microdissection.

2.2.1 Haematoxylin and eosin staining

Middle slides were dipped in a slide holder containing hematoxylin for 3 minutes, and the slides were transferred in a tray containing tap water and rinsed with running tap water at low pressure for 5 minutes. Slides were then submerged into a tray containing eosin for 20 seconds, and dehydrated by immersing them in ethanol at 95 %, and 100 % for 3 minutes respectively. Finally they were treated with xylene for 5 minutes and the sections were covered with a coverslip slide using Permount medium.

Haematoxylin is an alkaline salt that combines specifically with the negative charged phosphate group of nucleic acids, therefore colouring the nucleus blue. Eosin in contrast combines with positively charge cytoplasmic proteins, colouring the cytoplasm and collagen fibres pink.

2.2.2 Methyl green staining

Given that haematoxylin-eosin staining can interfere with PCR amplification, methyl green staining was used for microdissection. Two slides for each sample were submerged in a slide holder containing a 0.1% methyl green solution for 3 minutes, then transferred to a new tray containing distilled water for 5 minutes, and this step was repeated twice using each time clean water. Before microdissection the slides were air dried for 1 hour. Given that CTVT cells were simple to separate from host tissue, manual micro-dissection under stereomicroscopic observation was used.

The tip of a 25 gauge needle was dipped in a 1x PCR buffer solution, and the selected area containing more then 70-80 % tumour cells were scraped, then the microdissected cells were transferred to a 0.5 ml sterile microcentrifuge tube containing 50 µl extraction buffer: 1x GeneAmP PCR buffer II (without MgCl2) gold buffer, 0.5% tween 20, 0.4 mg/ml of proteinase K (QIAGEN). The micro-dissected samples were then incubated at 55 ^{0}C for 2 days (proteinase K was added after 24 hours). After 48 hours samples were heated at 99 ^{0}C for 15 minutes to inactivate the proteinase K.

2.3 Genomic DNA extraction

2.3.1 Blood and Tumour DNA extraction

DNA from tumour and blood samples was extracted using a QIAamp DNA kit (QIAGEN). Given that the QIA amp columns can bind up to a 100 µg of DNA, blood samples were divided in aliquot of 300 µl, whereas tumour biopsies were cut to a 1-cm^3 size and subsequently into small pieces for each column. The cells were lysed by adding 300 µl of buffer AL for blood samples and 300 µl of buffer ATL for tumour samples, which contain lysis buffer and 40 µl of proteinase K 20mg/ml (included in the kit). Samples were then incubated overnight at 55 ^{0}C. The next day, given that the QIA amp columns bind both RNA and DNA, 40 µl of RNAse A 20mg/ml (included in the kit) were added and the samples were incubated at room temperature for 3 minutes. After the addition of 200 µl of buffer AL, the samples were incubated at 70 ^{0}C for 10 minutes. To precipitate the DNA, 350 µl of 100% ethanol was added and mixed. Then the mixture was applied to the QIA amp columns and centrifuged at 8000 rpm for 1 minute. To wash the columns, 500 µl of buffer AW was applied and centrifuged for 1 minute at 8000 rpm, and the washing step was repeated again with a centrifugation period of 3 minutes, to remove all residues from the column. To elute the genomic DNA from the columns, 200 µl of preheated buffer AE was added and after incubation at room temperature for 2 minutes, and the columns were centrifuged at 8000 rpm for 2 minutes. The concentration of the DNA was determined using Nanodrop.

2.3.2 Isolation of total RNA

Total RNA was isolated from tumour and blood samples using Trizol Reagent (GIBCO BRL), which contains a mono-phasic solution of phenol and guanidine isothiocyanate. Tumour tissues were homogenised in 2 ml of Trizol Reagent with a homogeniser. The sample was then incubated for 10 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. Tumour lysates were subdivided in aliquots of 1 ml. Then 0.2 ml of chloroform was added to each tube and mixed vigorously by hand for 1 minute and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 g for 15 minutes at 4^0C. After centrifugation the RNA present in the upper aqueous phase was transferred to a fresh tube and precipitated by adding 0.5 ml of isopropyl alcohol. After a further incubation at room temperature for 10 minutes the samples were centrifuged at 12,000 g for 10 minutes. After removal of the supernatant the pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 3 minutes. The pellet was air dried and then resuspended in 20µl of RNAse-free water.

2.3.3 Purification of mRNA from total RNA

mRNA was isolated from total RNA using the Oligotex kit (QIAGEN). This procedure allows the isolation of poly-A mRNA by its hybridisation to T oligonucleotides covalently linked to polystyrene-latex spherical particles. 500 µg of total RNA were resuspended in 500 µl of RNAse-free water and transferred to a microcentrifuge tube containing 500 µl of pre-heated (70^0C) OBB buffer and incubated at 70 ^{0}C for 3 minutes to allow the disruption the RNA secondary structure. The tube was then incubated at room temperature for 10 minutes to allow hybridisation of the mRNA poly A tail with the oligo dT of the Oligotex particles. The particles were pelleted by centrifugation at 14,000 rpm for 2 minutes, and the supernatant was removed. The pellet was resuspended in 400 µl of buffer OW2 by vortexing and transferred to a column, and then centrifuged at 14,000 rpm for 1 minute. The Oligotex/mRNA particles were washed once with 400 µl of buffer OW2 and centrifuged for 1 minute at 14,000 rpm. The mRNA was eluted from the

Oligotex particle by adding 70 µl of pre-heated (70^0C) buffer OEB and centrifuged for 1 minute at 14,000 rpm. The quality and concentration of the mRNA was determined using a Bioanalyzer.

2.4 Genomic amplification

In order to analyse the genetic relationship between tumours and hosts and between tumours, several independent genetic markers were used. Genomic amplification was carried out using 50-100 ng DNA in 50 µl volume containing:1X buffer (10 mM Tris-HCl (pH 9), 100 mMNaCl, 01% triton X) , 1.5-2.5 mM $MgCl_2$, 200 µM dNTPs, 5 units of Taq DNA polymerase,10-20 pmol of forward and reverse primers, water (nuclease free) up to 50 µl. For microdissected samples forward primers were labelled with Cy5 and PCR products were run by non-denaturing polyacrylamide gel electrophoresis.To clone the PCR product, after 35 cycles of amplifications, 30-50 µl of PCR reaction were run in 1.5-2 % agarose gel and processed as described in the following sections.

Table 2.3 PCR primers

Primer name	Primer sequence	Product Size (bp)
LINE-1A	GGTGGGGCAGGGAGACAACATTTTA	390
	ATCCTAGAGAAGAACACAGGCAACAC	
LINE-1B	GGTGAGGCTTTCCCATCCTT	150
	CTTCTTGCAAGATACATCCA	
DLA-88exon 1-2-3	AGTCCAGCGGCGACGGCCAGTGTCCCCGGA	1,100
	AGCCCTCCCTAGTGGAGGCGAGATCGGGGA	
DRB1 exon 2	CCGTCCCCACAGCACATTTC	350
	TGTGTCACACACCTCAGCACCA	
DQB1	TCACTGGCCCGGCTGTCTCC	350
	GGTGCGCTCACCTCGCCGCT	
DQA1	CTCAGCTGACCATGTTGC	300
	GGACAGATTCAGTGAAGAGA	
DLA-88 exon 2	TCTCACCCGTCGGCTCCGCAG	350
	GATGGGGGTCGTGCCCTGGCC	
DLA-88 exon 3	ATTGGCGGCCTGTCGGG	350
	AGGCGAGATCGGGGAGGC	
CTVT DLA-88α	CCTTCAAGGAGACCGCACGAGGG	420
	CTCCAGGTAGTTCCTTTCGTGC	
CTVT DLA-88β	CCATCAAGGAGACCGCACAGGTG	420

	CTCCAGGTAGTTCCTTTCGTGC	
CTVT DRB1	CGGTTTCTGGCGAGAAGCA	150
	TCCACCGCGGCCCGCTCCTG	
CTVT DQB1	GGCTTCTGGCGAGAGACATC	150
	CGCCTCTGCTCCAAGAGCT	
CTVT DQA1	GAATTTGATGGCGATGAGTT	150
	TCAGGATGTTCAAGTTTTGTTTTAT	
SRY	CTCGCGATCAAAGGCGCAAGAT	80
	TTCGGCTTCTGTAAGCATTTTC	
650	GTCCTGGGTTCGGGTTAGTGTTAG	650
	GTCCTGGGTTGAAGCCCTACATTG	
H15422/L16106	CTCTTGCTCCACCATCAGC	722*
	AAACTATATGTCCTGAAACC	
H15422/H15710	CTCTTGCTCCACCATCAGC	290†
	GCATGGTGATTAAGCCCTTAT	

* for fresh tissues, † for paraffin embedded tumour.

Table 2.4 PCR conditions

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extention
LINE-1A	94 ^{0}C (5min)	94 ^{0}C (30sec)	60 ^{0}C (30sec)	72 ^{0}C (30sec)	72 ^{0}C (5min)
LINE-1B	94 ^{0}C (5min)	94 ^{0}C (30sec)	50 ^{0}C (30sec)	72 ^{0}C (30sec)	72 ^{0}C (5min)
DLA-88exon 1-2-3	95 ^{0}C (15min)	94 ^{0}C (1min)	65 ^{0}C (2min)	72 ^{0}C (3min)	72 ^{0}C (5min)
DRB1 exon 2	94 ^{0}C (5min)	94 ^{0}C (45sec)	61 ^{0}C (1min)	72 ^{0}C (1min)	72 ^{0}C (5min)
DQB1	94 ^{0}C (5min)	94 ^{0}C (5min)	66 ^{0}C (45sec)	72 ^{0}C (1min)	72 ^{0}C (5min)
DQA1	94 ^{0}C (5min)	94 ^{0}C (5min)	48 ^{0}C (1min)	72 ^{0}C (1min)	72 ^{0}C (5min)
CTVT DLA-88α	95 ^{0}C (15min)	94 ^{0}C (1min)	65 ^{0}C (2min)	72 ^{0}C (45sec)	72 ^{0}C (5min)
CTVT DLA-88β	95 ^{0}C (15min)	94 ^{0}C (1min)	64 ^{0}C (1min)	72 ^{0}C (45sec)	72 ^{0}C (5min)
CTVT DRB1	94 ^{0}C (5min)	94 ^{0}C (1min)	60 ^{0}C (45sec)	72 ^{0}C (45sec)	72 ^{0}C (5min)
CTVT DQB1	94 ^{0}C (5min)	94 ^{0}C (1min)	58 ^{0}C (45sec)	72 ^{0}C (45sec)	72 ^{0}C (5min)
CTVT DQA1	94 ^{0}C (5min)	94 ^{0}C (1min)	50 ^{0}C (45sec)	72 ^{0}C (45sec)	72 ^{0}C (5min)
SRY	94 ^{0}C (5min)	94 ^{0}C (1min)	62 ^{0}C (1min)	72 ^{0}C (30sec)	72 ^{0}C (5min)
650	94 ^{0}C (5min)	94 ^{0}C (1min)	65 ^{0}C (1min)	72 ^{0}C (1min)	72 ^{0}C (5min)
Mt-DNA/16106	94 ^{0}C (2min)	94 ^{0}C (15sec)	50 ^{0}C (30sec)	72 ^{0}C (1min)	72 ^{0}C (5min)
MtDNA/15710	94 ^{0}C (2min)	94 ^{0}C (15sec)	50 ^{0}C (30sec)	72 ^{0}C (30sec)	72 ^{0}C (5min)

2.5 Microsatellite genotyping

To analyse the genetic distance between tumours and hosts, nine microsatellite markers were chosen for their high polymorphism and their reliability for canine parentage testing. The markers are distributed on different chromosomes, except AHT125 and FH2010, however these two markers are distant enough to allow for independent recombination. Forward primers were labelled with different dyes (Table 2). To optimise the amplifications, a gradient PCR with different primer concentrations was performed. All PCR amplifications were carried out in 10 µl containing: 25ng of DNA, 2 mM $MgCl_2$, 100 µM each dNTP, 15 mM Tris-HCl (pH 8.3) 75 mM KCl, 0.5 units of AmpliTaq DNA polymerase (PE Applied Biosystems), and 0.3 –0.6 µM of primers (see Table 1) using a DNA Tetrad Engine (MJ Research). Cycling conditions consisted of: 5 min at 94 ^{0}C, followed of 30 cycles of 94 ^{0}C at 45 sec, annealing temperature (specific for each marker see table 1) for 45 sec, 1 min at 72 ^{0}C, followed of 5 min at 72 ^{0}C. Dye-labelled amplicons were pooled (3µl each) for multiplex analysis and diluted in 50 µl of deionised water and then 10 µl were mixed with 1µl of internal size standard GeneScan 350 TAMRA and 1x Loading dye (95 % formamide, 25 mM EDTA containing 50 mg/ml of dextran blue). The mixture was denatured at 95 ^{0}C for 3 min. PRC products were run for 2 hours on an ABI 377 sequencer gel electrophoresis apparatus (PE Applied Biosystems). Genotypes were analysed using GeneScan software (PE Applied Biosystems). To determine the specific tumour genotype the normal contaminating alleles were excluded.

Table 2.5 List of Microsatellite primers *

Marker	Ch	Repeat	Dye	Primer sequence forward and reverse	Prim (µM)	AT
AHT 125	24	CA	HEX	CCACGATCCTCATGTCACCT	0.4	60 ^{0}C
				ATCAAAGTCATGAAATTCCGTG		
CPH2	32	CA	FAM	TTCTGTTGTTATCGGCACCA	0.3	60 ^{0}C
				TTCTTGAGAACAGTGTCCTTCG		
CPH5	17	CA	HEX	TCCATAACAAGACCCCAAAC	0.6	58 ^{0}C
				GGAGGTAGGGGTCAAAAGTT		
CPH6	23	CA	FAM	CATTGGCTGTTTGACTCTAGG	0.5	60 ^{0}C
				ACTGATGTGGGTGTCTCTGC		
CPH9	29	CA	TET	CAGAGACTGCCACTTTAAACACAC	0.3	62 ^{0}C
				AAAGTTCTCAAATACCATTGTGTTAA		

FH2001	23	GATA	TET	TCCTCCTCTTCTTTCCATTGG	0.3	54
				TGAACAGAGTTAAGGATAGACACG		
FH2004	11	GAAA	TET	CTAAGTGGGGAGCCTCCTCT	0.3	60
				ACTGTGACCTACTGAGGTTGCA		
FH2010	24	ATGA	FAM	AAATGGAACAGTTGAGCATGC	0.3	62
				CCCCTTACAGCTTCATTTTCC		
FH2054	12	GATA	HEX	GCCTTATTCATTGCAGTTAGGG	0.4	56
				ATGCTGAGTTTTGAACTTTCCC		

* The microsatellites used in this study. The primer sequences are from the dog genome project web site (www.ihere.org/scienc/dog_genome/dog.html).

In order to determine the genetic origin of the tumour and to estimate its age, tumours were analysed using 96 dinucleotide markers used previously to determine the genetic relationship among 85 different dog breeds. List of primers sequences is shown in Table 2.6. Amplifications were performed using M13 tailed forward primers containing 19 bases of M13 sequence on the 5' end of the specific microsatellite primers, and the PCRs were labelled using tagged M13 primers. In order to run in the same capillary 4 different amplicons, M13 primers were tagged with either 6FAM, VIC, NED, PET dyes. PCRs were carried out in 5 µl of volume containing: 0.3 µM of forward (M13tailed) and reverse primers , 1 X PCR buffer, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 0.15 units of Taq DNA Polymerase (Bioline, USA), 2 ng of genomic DNA, and 0.25 pmol of M13 labelled primer. Reactions for microdissected samples were carried out in 10 µl volume. PCRs were performed in 96 well plates on the MJ research PTC 200 thermal cycler using the following conditions: 94 ^{0}C x 5 minutes, 35 cycles 94 ^{0}C x 20 seconds, 58 ^{0}C or 50^{0}C x 20 seconds, 74 ^{0}C x 20 seconds, and a final extension of 74 ^{0}C x 5 minutes.

Following amplification, the four differently labelled reactions were mixed. 10 µl of the pooled PCRs was mixed with 1 volume of deionised formamide and 15 pmol of GeneScan-500LIZ size standard (ABI, Foster, CA). Samples were run on an ABI 3730 capillary electrophoresis instrument. The alleles were analysed using GeneMapper 3.0 (ABI, Foster, CA). To align the previously reported genotypes (414 dogs of 85 different breeds listed in table 2.7) with the tumour genotype, a common positive control was included in each plate.

Table 2.6 microsatellite primers (Parker et al, 2004).

Marker Number	Marker Name	Chromosome	M13 tagged forward and reverse primers;	Annealing Temperture	Published PIC*
1	REN285G14	1	CACGACGTTGTAAAACGACAACATGGTGACGAGAAGGCT	55	NA
			CCATTCGCCACAAGTAGGTT		
2	C01.673	1	CACGACGTTGTAAAACGACCCACCACTTACATAAACATGGC	58	0.36
			TTCCAGACTGCTGCCTCC		
3	REN112I02	1	CACGACGTTGTAAAACGACATAGCCCATGAAATCCA	58	0.76
			CCCCAAATACATCCCTACAT		
4	REN172C02	1	CACGACGTTGTAAAACGACCCCTTAGGAGGAGGCAAGAC	55	0.48
			CGTGCTTTGTTATGGCTTGA		
5	FH2793	1	CACGACGTTGTAAAACGACCTATGTGCACGCTGAGAGAG	58	0.76
			TACCCATAAAGTTGGGCTTG		
6	REN143K19	1	CACGACGTTGTAAAACGACGTCAGAGCCCCAGAGACAAG	55	0.5
			CTTTCTTCCGCCACTACCTG		
7	FH2890	2	CACGACGTTGTAAAACGACCCAGATTAACCAGGATGAGG	55	0.59
			AATGGCAAGGATGCTACTCC		
8	C02.466	2	CACGACGTTGTAAAACGACTCTGGATTGTGGTCACAACC	58	0.55
			ACTGGACACTTCTTTTCAGACG		
9	C02.894	2	CACGACGTTGTAAAACGACTCAGCATCTAGAAAATTAGGT	58	0.72
			ACTCATTTTCTCTTATTCTGCAG		
11	FH2895	3	CACGACGTTGTAAAACGACTGCGATACACTTAGAAAACAGG	58	0.7
			AAAAACCCACAACAAAGTGC		
12	REN157C08	3	CACGACGTTGTAAAACGACGCAAATGGCAAGATTTCGTT	55	0.72
			CAAGGGTTAGCACCTGGGTA		
13	C03.445	3	CACGACGTTGTAAAACGACTCTTTGGTAAAGTCTCCATGGG	58	0.6
			GGGTGTGAAAACAGCCAACT		
14	FH2732	4	CACGACGTTGTAAAACGACGAAAGGGTGTGGATAAAGAGC	58	0.84
			TGGCATATTCAACAAATTGC		
15	FH2776	4	CACGACGTTGTAAAACGACGGAACAGATGAGAAGCATGG	58	0.49
			CTGGGTGGTTCAGTAGTTGG		
16	REN160J02	4	CACGACGTTGTAAAACGACACCGTGCACAAGTCAGTCAG	58	0.82
			AAGCTGAGCCATTCTTTTCC		
17	REN262N08	4	CACGACGTTGTAAAACGACAGGATTTTGTGGTGATTGGG	55	NA
			TTGCAGCCTATTGTGGACTTT		
18	REN92G21	5	CACGACGTTGTAAAACGACTACTCGTTCTGTGCCATTTCT	58	0.66
			CACACCAGCTCGTCCTCATAA		
19	REN285I23	5	CACGACGTTGTAAAACGACCAGCTGTCCGGGGATATAAA	55	0.58
			GGCTGTGGTTTGTCCTTGTT		
20	C05.414	5	CACGACGTTGTAAAACGACGCCCCTATTTTTTTTTTTCCC	58	0.47
			TTGGGTTTCACACTCAGCAG		
21	FH2752	6	CACGACGTTGTAAAACGACTTTAGGCATTTGAGGAGGAG	58	0.38
			GGCACAGAATCCAACTTGAG		
22	REN210I14	6	CACGACGTTGTAAAACGACCTGCTCTCTCCCCCAACTTA	55	0.66
			CAGGGCCATTGGTCTAGAAA		
23	REN37H09	6	CACGACGTTGTAAAACGACATTCCCTTGTATTGCTCA	58	0.67
			CCCCAAAAAATCCAACCA		
24	REN97M11	7	CACGACGTTGTAAAACGACTGAGGCTCCGTGGGTATGTG	55	NA
			AGGCCAAGGGAATGATGCTC		

25	REN286L19	7	CACGACGTTGTAAAACGACCAGTAATGTTTTGTGGCCTCTG	58	0.66
			GGGAGTGGGGGAAATAAATG		
26	FH2860	7	CACGACGTTGTAAAACGACTGCCCTCAAACAATTTGC	55	0.62
			ATCATCCTAGCACTCAGAAGG		
27	REN204K13	8	CACGACGTTGTAAAACGACTCGGGATGTTTCTCTTCCAC	55	0.48
			CTGCTTAAATTCTCCCAGCG		
28	C08.373	8	CACGACGTTGTAAAACGACTATTTAAAAAATCCCAGGCACA	58	0.68
			AGCATCAATTAGATGTCAGCG		
29	C08.618	8	CACGACGTTGTAAAACGACCAACCCAGGGTGGAAGC	55	0.82
			TAGCAAGAAAATGTGCCCA		
30	C09.173	9	CACGACGTTGTAAAACGACATCCAGGTCTGGAATACCCC	58	0.78
			TCCTTTGAATTAGCACTTGGC		
31	C09.474	9	CACGACGTTGTAAAACGACTTAAGCCTTATTTTGTGTTGGG	55	0.78
			TCCAGGAAGTGTCTGCAGG		
32	FH2885	9	CACGACGTTGTAAAACGACCTTTTAGGGTGCCTTCAACC	55	0.74
			TGGATTATTAAGGGGAATTTAGC		
33	C10.781	10	CACGACGTTGTAAAACGACACCTCCAAGATGGCTCTTGA	55	0.62
			ACGTCGAGCTCCTGGCAT		
34	REN73F08	10	CACGACGTTGTAAAACGACATTCCCCAGCGATACCA	55	0.54
			AACAGCATTTCAGACAGAGG		
35	REN154G10	10	CACGACGTTGTAAAACGACTGGACGCTAAGCCTGACTTT	55	0.71
			AAACGGAGTTCCATCTCTGG		
36	REN164B05	11	CACGACGTTGTAAAACGACTGGGATGTGTGTCATGTGTG	55	0.5
			CCTATGCAGGGTAGGCACAT		
37	FH2874	11	CACGACGTTGTAAAACGACAACTTCTGGCTTTCATACCG	55	NA
			TCTTCAAAGAAAACCAACAGG		
38	C11.873	11	CACGACGTTGTAAAACGACCTGGCAGATTACAGGTAGC	58	0.81
			GTTCTCCAAAGCACTCAT		
40	REN213F01	12	CACGACGTTGTAAAACGACCATTCTGGGCAGGTTTCATT	55	0.82
			GGGGACAAATTTCCACTCCT		
41	REN208M20	12	CACGACGTTGTAAAACGACTGCATCATTTGTGGGTGTTT	58	0.64
			ATGGAGCATGGGTGAGAAAT		
42	REN94K11	12	CACGACGTTGTAAAACGACATACCACTGTCCCTCCTCTTA	55	0.56
			ATCCTGGTATCAAATCTATCA		
44	REN286P03	13	CACGACGTTGTAAAACGACGCACATTCACAAAGTGGTGC	58	0.78
			GCAATGGAAAGAGGATGGAA		
45	C13.758	13	CACGACGTTGTAAAACGACAAGCATCCAGAATCCCTGG	55	0.75
			GTTGATTGGGAGATAATCCACA		
46	C14.866	14	CACGACGTTGTAAAACGACTGTCATAATAGTTGGAATGAC	55	0.74
			TTAGAGCTTACTCATGATATCTG		
47	FH3072	14	CACGACGTTGTAAAACGACCCTGGATTAACAGTTGTCTGG	55	0.63
			CCTGGATTATAAGCATGAGAGC		
48	FH3802	15	CACGACGTTGTAAAACGACTTTTCACCAGCTCTGAGATAGC	55	0.44
			TTTCTGAGCCACTTTTCCATAG		
49	REN06C11	15	CACGACGTTGTAAAACGACTGCAGGGCAGAGGCTGGAGG	58	0.79
			GGGGGTGTCGGTGGAGTTCT		
50	REN144M10	15	CACGACGTTGTAAAACGACTCTCATGCAAATCTTCGTTCA	58	0.66
			GCCACCTCATTCCAAAAAGA		
51	REN85N14	16	CACGACGTTGTAAAACGACAAGGCAGGAGGAGGAGCAC	58	0.78

			TATGGAGATGGAGGGCACAC		
52	FH3096	16	CACGACGTTGTAAAACGACTCAGCTCAGGGAGTGATCC	55	0.79
			ATGAGGAGGTGCAACTATCC		
53	C17.402	17	CACGACGTTGTAAAACGACAAATGGGTAATTCATCCAGTGC	58	0.75
			CAGGCTTTGTTGAGGTGTCA		
54	REN50B03	17	CACGACGTTGTAAAACGACATGTGGGGTAAAATAAAGG	58	0.74
			AACACTGACATGCATCCAC		
55	REN112G10	17	CACGACGTTGTAAAACGACGAGGGATGGCTGTCAAGA	55	0.7
			TGGTGGATAAATAGATAAGGA		
56	REN186N13	18	CACGACGTTGTAAAACGACAGGCTCTCCGAGGGTAAGAC	58	0.66
			ATTGCTTGGATAAGAGGGGG		
57	FH2795	18	CACGACGTTGTAAAACGACGGCTGGACTTTTGTCATTTG	58	0.71
			TGTGAGTAGGGTAGGGCAAG		
58	C18.460	18	CACGACGTTGTAAAACGACCTTCCCATTATAGCCCTGTCC	58	0.53
			GGTGTCAGGAAAATGAGACCA		
59	FH2783	19	CACGACGTTGTAAAACGACTCCCTCCTGACACTGCTTTA	55	NA
			AGGCCTGCTGTTTCTCTTCT		
60	REN91I14	19	CACGACGTTGTAAAACGACCCCTTTTGCACTACTGTTGAG	58	0.72
			TCCCTTTTTGTGGCTGAA		
61	REN274F18	19	CACGACGTTGTAAAACGACTTCTCGTGACCCCTAAAGGA	58	0.66
			GGCATAAATTGTCTTTGCCC		
62	FH2887	20	CACGACGTTGTAAAACGACAAACAAGACAGTAGGAAGAGAGG	55	0.77
			CAAGATTCAAAACAAGCAACC		
63	FH3109	20	CACGACGTTGTAAAACGACTGGGAGCTTCATACAAATGC	58	0.62
			CCATGGAGAGTGGTTATTGC		
64	REN293N22	20	CACGACGTTGTAAAACGACAATTACTCGGCCTCTCTGGG	58	0.48
			AATGACATTGAGCCTGGGAA		
65	FH2914	21	CACGACGTTGTAAAACGACGTGATCCACTTGCTTGTATCC	55	0.61
			ATAGCCTTGGGAATTTTTGC		
66	FH3069	21	CACGACGTTGTAAAACGACCATGCCTGACTCAACTGATG	55	0.53
			TATGGACCTTCGTTCAGAGG		
67	REN49F22	22	CACGACGTTGTAAAACGACGGGGCTCTGTTATTAGGTG	55	0.66
			TCATAAGGCAAAGAAAACC		
68	REN107H05	22	CACGACGTTGTAAAACGACTGACTTACTCTAGCCACTTTT	55	0.86
			TTGTCCCTTGTATAACTGATG		
69	REN78I16	22	CACGACGTTGTAAAACGACTTACATTTAGGGGCTCCAGT	55	0.63
			TTAGACAAAATAGGCTTCAA		
70	FH3078	23	CACGACGTTGTAAAACGACGCCTTCTTTGGAAAAACACC	55	0.67
			TTCAGGGAATTCTTTCTTGG		
71	C23.277	23	CACGACGTTGTAAAACGACACACATTTGTGTGCTTGTCTTG	55	0.54
			TTGTATGGAGGTGGGGAGAG		
72	REN181K04	23	CACGACGTTGTAAAACGACACAAGCCGACTCTAGCGAAA	58	0.64
			AGATGGGGCCTAACCAAAGT		
73	REN106I06	24	CACGACGTTGTAAAACGACGGTCCCATCCTCAAAATCCTC	55	0.58
			CCCTTCTGGCCTCCTACACA		
74	FH3083	24	CACGACGTTGTAAAACGACATTTGCCAGGTACCATTCC	55	0.61
			CAGGTTATTCTGGGCTATGG		
75	REN54E19	25	CACGACGTTGTAAAACGACGCAGACGAGCACACCGAA	55	0.54
			TATTCCACATCATTCACC		

77	REN87O21	26	CACGACGTTGTAAAACGACTGGCTGTGGCTAAGGCTTTGT	55	0.62
			CCCCTTCCAGCTTCGGTGTAG		
78	C26.733	26	CACGACGTTGTAAAACGACCCCTCTACTTATGTCTCGGCC	55	0.61
			GAGAGGAGAAACAACCAACACC		
79	C27.442	27	CACGACGTTGTAAAACGACCCAAGAACAGCCTAAGCTGG	55	0.74
			ACACATACACGCCCAATTCA		
80	C27.436	27	CACGACGTTGTAAAACGACCAGCTGGATTGGGGACTC	55	0.51
			CATCTTGCTCTCTCAAC		
81	REN72K15	27	CACGACGTTGTAAAACGACCCGATGCCTGTCCTTTGA	55	0.66
			ACCTGGCCTCTTCTGTTGTCT		
82	FH2759	28	CACGACGTTGTAAAACGACAGTACTTGAGGCTTGGAGTCAG	55	0.71
			CAAGCTGAGAGCCATGTAGG		
83	FH2785	28	CACGACGTTGTAAAACGACATGGCAGGTCAAGAGTATGG	55	0.46
			GATAGATCCAAGCCAACACC		
84	REN239K24	29	CACGACGTTGTAAAACGACTTCATTGGCTGGTGACTTTG	55	0.78
			CCCCAGGACCATTTGTTAGA		
85	FH3082	29	CACGACGTTGTAAAACGACAGTAAAGGGTTCTCAAGTGTGC	55	0.54
			CATTTGTCATTGTGGAAAACC		
86	REN51C16	30	CACGACGTTGTAAAACGACCAGTTCATCCTTCCCCCTCTC	55	0.8
			GTGCTAGTCTGGCTGTGCTCA		
87	FH3053	30	CACGACGTTGTAAAACGACGATTAAAAGGGCAAGCAACC	55	0.74
			TCATCTCCAGCTTTTCATGG		
88	REN43H24	31	CACGACGTTGTAAAACGACCAGTGAGCAAAGCAAATGAA	55	0.66
			ATGTGAACCCCGCCCAATA		
89	FH2712	31	CACGACGTTGTAAAACGACAAGGTAGTCCCACGATCCTC	55	0.67
			GAGCCCTGTTCTCAGGTTG		
90	FH2875	32	CACGACGTTGTAAAACGACTGATACCCATTAAGTCCATCC	55	0.6
			CAATACCCTGATACCAAAACC		
91	FH2790	33	CACGACGTTGTAAAACGACCCAATATTGTTAAGAAGTTCAAGC	55	0.58
			AGGCCTTCTCTGTCCTCTTG		
92	REN291M20	33	CACGACGTTGTAAAACGACCCTCTAGATCCATCCATATTGTCA	58	0.76
			TGTCCACCCACAGATGAATG		
93	REN160M18	34	CACGACGTTGTAAAACGACAATGGTGGTGATATTCACAGAGA	58	0.76
			GGATGCTTGGGAATCTTGAA		
94	FH3060	34	CACGACGTTGTAAAACGACAGGCAGAGCTAAACCTGAGC	55	0.4
			TACAGGCACTCCTTCCTACG		
95	REN314H10	34	CACGACGTTGTAAAACGACGAAATTGTTCCATTTCTGTGACAT	55	0.54
			AGGTTTGGGCTCCTCATTCT		
96	REN01G01	35	CACGACGTTGTAAAACGACTACATCTCCACATCTACTGA	55	0.54
			GAGACTTAACACAGTATTTG		
97	REN112C08	35	CACGACGTTGTAAAACGACATGGCCCACCGATACACA	55	0.42
			TCGGGGACATACTTGAACC		
98	REN106I07	36	CACGACGTTGTAAAACGACTTCCCCAAGCCACACC	55	0.78
			AACCACTATCCAACTTTAT		
99	FH2708	37	CACGACGTTGTAAAACGACCAGCAATTGGACAAGAAAAAG	55	0.63
			GAAAGAGGATGAAGGGTGTG		
100	REN86G15	38	CACGACGTTGTAAAACGACCAGCGAATTTGGGCACTAA	55	0.76
			CAACTAAGGCAGAGAATACCA		

* PIC = Polymorphism information content

Table 2.7 List of breeds used to analyse the genetic origin of the CTVT

Afghan Hound	Irish Setter
Airedale Terrier	Irish Terrier
Akita	Irish Wolfhound
Alaskan Malamute	Italian Greyhound
American Cocker Spaniel	Keeshond
American Water Spaniel	Kerry Blue Terrier
American Hairless Rat Terrier	Komondor
Australian Shepherd	Kuvasz
Australian Terrier	Labrador Retriever
Basenji	Lhasa Apso
Basset Hound	Manchester Terrier Toy
Beagle	Mastiff
Bedlington Terrier	Miniature Bull Terrier
Belgian Sheepdog	Miniature Schnauzer
Belgian Tervuren	Newfoundland
Bernese Mountain dog	Norwegian Elkhound
Bichon Frise	Old English Sheepdog
Bloodhound	Pekingese
Border Collie	Pharaoh Hound
Borzoi	Pointer
Boxer	Pomeranian
Bulldog	Portuguese Water Dog
Bullmastiff	Presa Canario
Cairn Terrier	Pug Dog
Cavalier King Charles Spaniel	Rhodesian Ridgeback
Chesapeake Bay Retriever	Rottweiler
Chihuahua	Saint Bernard
Chow Chow	Saluki
Clumber Spaniel	Samoyed
Collie	Schipperke
Dachshund	Shar Pei
Doberman Pinscher	Shetland Sheepdog
English Cocker Spaniel	Shiba Inu
Flat-coated Retriever	Shih Tzu
French Bulldog	Siberian Husky
German Shepherd	Soft-coated Wheaten Terrier
German Shorthaired Pointer	Standard Poodle
Giant Schnauzer	Standard Schnauzer
Golden Retriever	Tibetan Terrier
Great Dane	Welsh Springer Spaniel
Greater Swiss Mountain dog	West Highland White Terrier
Greyhound	Whippet
Ibiza Hound	

2.6 Gene quantification by real time PCR

The polymerase chain reaction is a technique that allows the amplification of specific DNA sequences. The reaction is characterised by an initial logarithmic increase of the target sequence per cycle, followed by a plateau where there is no increment. The final amplified product is visualised by ethidium bromide after gel electrophoresis. The peculiarity of real time PCR is that the amplification process is monitored at every cycle in real time by measuring fluorescence signals that are proportional to the amount of the PCR product generated (Wilhelm and Pingoud, 2003). The fluorescence signals can be generated by sequence specific fluorescence probes (TaqMan), or by fluorescence dyes that bind double stranded DNA. The most used fluorescence dye is SYBR green 1 (Ponchel et al., 2003). It is an asymmetric cyanine dye with a DNA binding affinity 100 times higher then that of ethidium bromide. The fluorescence emitted by the bound dye is 1000 fold higher than that of the free dye. SYBR green is able to detect a single target molecule (Ponchel et al., 2003).

In order to determine the gene dosage of the DLA genes, real time PCR based on SYBR-Green I fluorescence was used. PCRs were carried out using the SYBR-Green PCR Core Reagents kit (Applied Biosystems). In order to avoid the amplification of non-specific products and reduce the formation of primer dimers, primer concentrations for each target and reference gene was optimised using PCR reactions containing 0.3, 0.5 and 1 μM of primers. To check for specific amplification, PCR products were analysed by agarose gel electrophoresis. After optimisation quantitative PCR reactions were carried out in 50μl volume containing 1X PCR buffer, 200μM dNTPs, 2μM $MgCl^2$. 0.25μl of AmpliTaq Gold DNA polymerase (5U/μl), 50ng and 100ng of genomic DNA for fresh and microdissected samples respectively. Given the presence of normal cells within the tumour tissue, to determine the tumour specific gene dosage, tissues with a high percentage of tumour cells must be used. The percentage of the tumour cell within the tumour tissue was determined by histological analysis, and all tumour samples analysed

contained more than 80% tumour cells. To normalize the SYBR green fluorescence variation due to pipetting variation and fluorescence fluctuation, an internal fluorescence dye (ROX) that does not interfere with the PCR reactions was included in the PCR buffer.

All quantitative PCRs were performed in the ABI Prism® 7000 Sequence Detection System (SDS). This system consists of a thermal cycler and a laser directed via optical cable fibres to each of the 96 sample wells. A device collects the emission of the fluorescence signal from each sample and the data are analysed automatically by SDS software to calculate the Ct value, melting curve and gene copy number. The threshold cycle (Ct) parameter is the cycle number at which the fluorescence passes a fixed threshold above the baseline. The threshold is calculated as ten times the standard deviation of the average baseline fluorescence signal measured between cycles 3-15. Therefore the fluorescence signal that is detected above the threshold representing the background is analysed to calculate the Ct value. The data are displayed as an amplification plot, in which the fluorescence signals are plotted against the number of cycles (see Fig 2.2a). Following the amplification, a melting curve is performed, in which the temperature is increased very slowly from 60 ^{0}C to 95 ^{0}C. At low temperature SYBR green binds to the double stranded PCR product, generating an high fluorescence signal, whereas at high temperature the denaturation of the PCR product increases the proportion of the free SYBR green, therefore decreasing the fluorescence signal (see Fig. 2.2b).

Target DNA can be quantified using either absolute or relative quantification. Absolute quantification determines the copy number of the target DNA, whereas relative quantification determines the ratio between the target and a reference gene present in the same sample. In this study I used the absolute quantification method. To determine DNA copy number an external standard curve was constructed. To generate the standard curve, target and reference genes were amplified and cloned

into the pGEM T vector. The copy number of standard DNA molecules was calculated using the following formula:

molecules/µl = (g/µl DNA /plasmid length x 660) x 6.022 x 10^{23}

PCR reactions containing dilution series from 10^2-10^9 copies of each plasmid were performed using the same conditions described above for each target and reference gene. The resulting Ct values were plotted in a linear line as a function of the log 10 concentration of input plasmid amount. The slope of the line was used to determine PCR efficiency and the unknown copy number in the samples. To determine the correlation between input data a linear regression analysis was automatically calculated (Fig 2.2c). For each target and reference gene, tumour and normal copy number was determined against a standard curve run in parallel with the samples. All samples were run in triplicate. Amplification of the correct sequence was confirmed by dissociation curve analysis. To determine the DLA gene dosage, two internal reference genes were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are housekeeping genes that are expressed normally in all cells and are unlikely to be mutated or deleted in tumours; therefore they are routinely used to normalise the amount and quality of genomic DNA and RNA.
The gene dosage (N) was determined using the formula (Bieche et al., 1998):

$$N = \frac{\textbf{Copy number of target gene}}{\textbf{Copy number of reference gene}}$$

To exclude the possibility that the gene dosage of reference genes were altered in tumour tissue, the ratio between GAPDH and β-actin was also calculated. Given that target-reference ratio is variable between different normal samples, to determine the cut-off values in the tumour samples, 4 normal samples were also tested.

Table 2.8 primers for q-PCR

Gene	Forward	Reverse	Size bp
GAPDH	GGCGGGGCCAAGAGGGTCA	TCTTGAGGGAGTTGTCAT	120
β-Actin	CTCCATCATGAAGTGTGACGTTG	CGATGATCTTGATCTTCATTGTGC	150
DQA1	CTCAGCTGACCATGTTGC	CACAGGCAGCCGCCAGAC	150
DQA1†	TAAGGTTCTTTTCTCCCTCTGT	TGCTAGGGAGGAAGGGGAAAG	389
DQB1	TCACTGGCCCGGCTGTCTCC	CTCCCCCACGTCGCTGTC	150
DRB1	CCGTCCCCACAGCACATTTC	TGTGTCACACACCTCAGCACCA	350
DLA-88	TCTCACCCGTCGGCTCCGCAG	GATGGGGGTCGTGCCCTGGCC	350
DRA	CATCCAAACCCCAGTGCTCC	ACCCCTGTGGAACTGGGAGAG	212

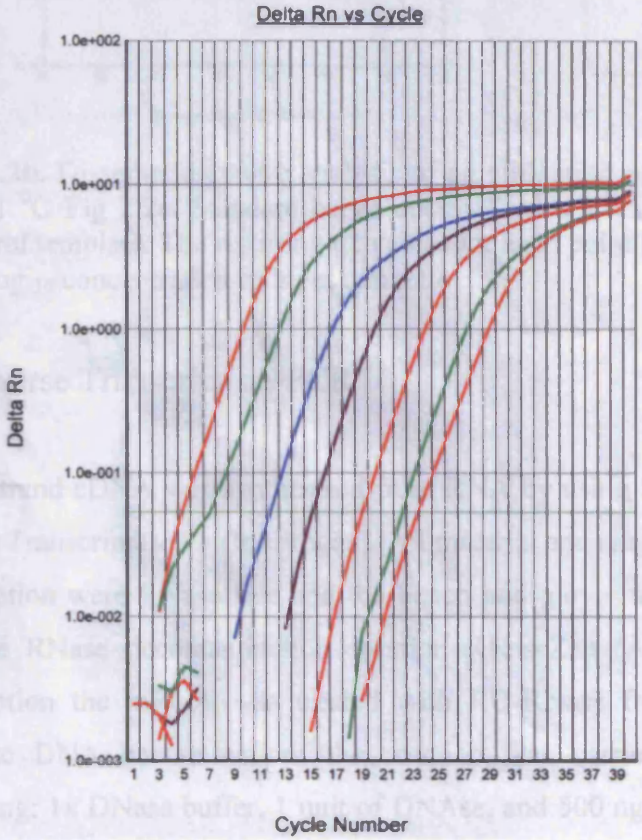


Fig 2.2a. Amplification plot of a dilution series containing 10^2-10^9 template copies

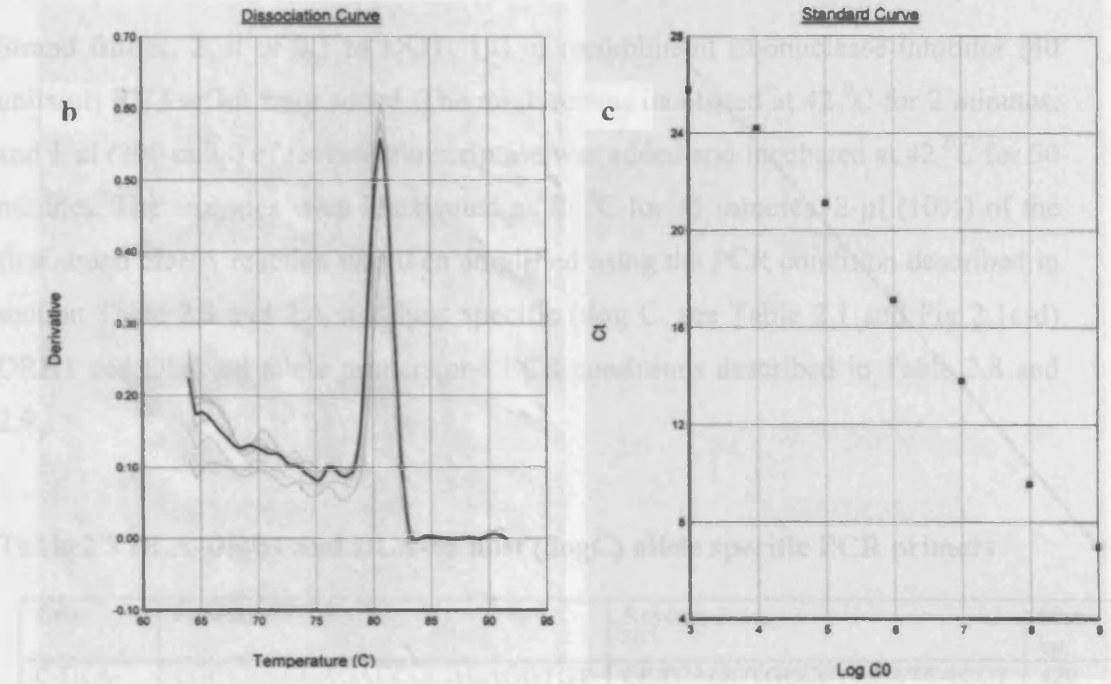


Fig2.2b. Dissociation curve analysis of amplification products with melting point of 81 ^{0}C **Fig 2.2c**. Standard curve obtained with a tenfold dilution of a positive control template. The resulting Ct values for each point are plotted as a function of the log $_{10}$ concentration of input template.

2.7 Reverse Transcription-PCR

Single strand cDNA was synthesised from RNA by using SuperScript II RNase H-Reverse Transcriptase kit (Invitrogen). All material and reagents used during reverse transcription were RNase free and the bench and gloves and pipettes were treated with the RNase decontamination solution RNaseZap (Ambion). Before reverse transcription the mRNA was treated with RQ-RNase free-DNase (Promega) to eliminate DNA contamination; the reaction was carried out in 10µl volume containing: 1x DNase buffer, 1 unit of DNAse, and 500 ng of mRNA. The reaction was incubated for 10 minutes at 37 ^{0}C, and then DNAse was inactivated by heating at 65 ^{0}C for 15 minutes. 50 ng of mRNA was transferred in a 0.5 microcentrifuge tube containing: 2 pmol of the specific reverse primers, 1 µl of 10 mM dNTP mix, and sterile water up to 12 µl. The reaction was incubated at 65 ^{0}C for 5 minutes to disrupt the mRNA secondary structure and to allow primers to bind the complementary target sequence, and then quickly chilled on ice. 4 µl of 5x First

Strand Buffer, 2 µl of 0.1 M DDT, 1µl of recombinant ribonuclease inhibitor (40 units/µl) RNAseOut were added. The mixture was incubated at 42 ^{0}C for 2 minutes, and 1 µl (200 units) of reverse transcriptase was added and incubated at 42 ^{0}C for 50 minutes. The enzymes were inactivated at 70 ^{0}C for 15 minutes. 2 µl (10%) of the first strand cDNA reaction was then amplified using the PCR condition described in section Table 2.3 and 2.4, and host specific (dog C, see Table 2.1 and Fig 2.1c-d) DRB1 and DLA-88 allele primers and PCR conditions described in Table 2.8 and 2.9.

Table 2.8 DLA-DRB1 and DLA-88 host (dogC) allele specific PCR primers

Gene	Forward	Reverse	Size bp
C-DLA-88 (29)	CGCCAAGGAGACCGCACAGGTGT	CCTCAGGTGCCCTGCATCACCT	420
C-DLA-88 (42)	CCATCAAGGAGACCGCACAGAGG	CCTCAGGTGCCCTGCATCACCT	420
C-DLA-DRB1	CGGTTCGTGGAAAGATACA	CAATCACCCCGTAGTTGTG	169

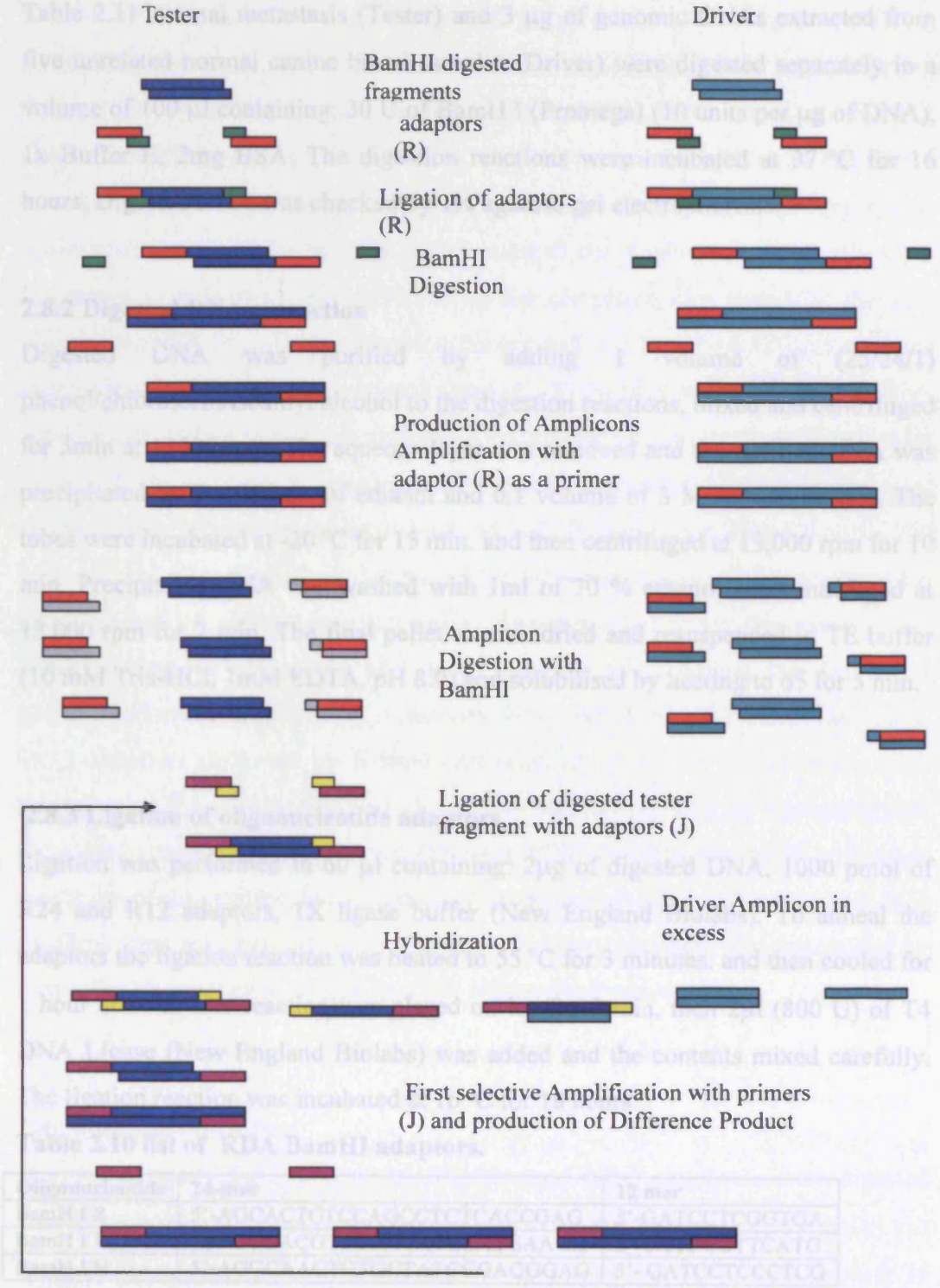
Table 2.9 PCR conditions for host (dog C) host allele specific DLA-DRB1 and DLA-88 amplifications

PCR steps	C DLA 88 (29)	C DLA 88 (42)	C DLA DRB1
Initial Denaturation	95 (3min)	95 (3min)	95 (3min)
Denaturation	94 (1min)	94 (1min)	94 (1min)
Annealing	65 (2min)	64 (1min)	61 (1min)
Extension	72 (45sec)	72 (45sec)	72 (45sec)

2.8 Representational difference analysis

In an attempt to identify unique DNA in CTVT possibly representing part of the genome of an oncogenic virus or microbe, representational difference analysis (RDA) was performed as outlined in Fig 2.4

Fig 2.3 Outline of RDA Process



2.8.1 Genomic DNA digestion

3 μg of genomic DNA extracted from a metastatic tumour removed from dog C (see Table 2.1) internal metastasis (Tester) and 3 μg of genomic DNAs extracted from five unrelated normal canine blood samples (Driver) were digested separately in a volume of 100 μl containing: 30 U of BamH I (Promega) (10 units per μg of DNA), 1x Buffer E, 2mg BSA. The digestion reactions were incubated at 37 °C for 16 hours. Digested DNA was checked by 1% agarose gel electrophoresis.

2.8.2 Digested DNA extraction

Digested DNA was purified by adding 1 volume of (25/24/1) phenol/chloroform/isoamyl alcohol to the digestion reactions, mixed and centrifuged for 3min at 13000 rpm. The aqueous layer was removed and the purified DNA was precipitated by 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate. The tubes were incubated at -20 °C for 15 min. and then centrifuged at 13,000 rpm for 10 min. Precipitated DNA was washed with 1ml of 70 % ethanol and centrifuged at 13,000 rpm for 2 min. The final pellet was air dried and resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and solubilised by heating to 65 for 5 min.

2.8.3 Ligation of oligonucleotide adaptors

Ligation was performed in 60 μl containing: 2μg of digested DNA, 1000 pmol of R24 and R12 adaptors, 1X ligase buffer (New England Biolabs). To anneal the adaptors the ligation reaction was heated to 55 °C for 3 minutes, and then cooled for 1 hour at 4 °C. The reaction was placed on ice for 3 min, then 2μl (800 U) of T4 DNA Ligase (New England Biolabs) was added and the contents mixed carefully. The ligation reaction was incubated at 16 °C for 18 hours.

Table 2.10 list of RDA BamHI adaptors.

Oligonucleotide	24-mer	12 mer
BamH I R	5'-AGCACTCTCCAGCCTCTCACCGAG	5'-GATCCTCGGTGA
BamH I J	5'-ACCGACGTCGACTATCCATGAACG	5'-GATCCGTTCATG
BamH I N	5'-AGGCAACTGTGCTATCCGAGGGAG	5'- GATCCTCCCTCG

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BamH I J	5'-ACCGACGTCGACTATCCATGAACG	5'-GATCCGTTCATG
BamH I N	5'-AGGCAACTGTGCTATCCGAGGGAG	5'- GATCCTCCCTCG

2.8.4 Production of amplicons

PCR amplification of ligated DNA was performed in 400 µl volume containing: 80ul of: 5X PCR buffer, 32 µl of 4 mM dNTPs, 500 pmol of R24 adaptor and100 ng of the R ligated DNA. 100 µl of mineral oil was layered on the top of the reaction mix. Initially PCR reaction was heated to 72 °C for 3 min to dissociate the R12 adaptors from the template, then 20 U of Taq polymerase (AmpliTaq Perkin-Elmer Cetus) was added under the oil layer and the reaction was mixed by pipetting up and down several times. The samples were incubated for 5 min at 72 °C to allow the fragments 3' ends to extend thus forming the complementary strand for the R24 oligonucleotides. In order to determine the optimal number of amplification cycles that yield a distinct and more intense band of 750 bp (due to the presence of high copy of a repetitive element) a preliminary amplification was performed; at cycles 25, 27, 30, 35, the amplification reaction was placed on ice and 10 µl was removed and run on a 1% agarose gel and visualised by UV illuminator. Given that the 750 bp band was more distinct and intense at the 30^{th} cycle, six tubes of tester (tumour) and thirteen of driver (normal) were amplified separately (to avoid DNA contamination between tester and driver samples while adding the Taq DNA polymerase) using the following conditions: 30 cycles of (95 °C 1 minute and 72 °C for 3 minutes) followed by a final extension at 72 °C for 10 minutes. After amplification the PCR profile was checked by agarose (1.5 %) gel electrophoresis. Once the mineral oil was removed from the PCR reactions, the tester and driver amplicons were purified and precipitated as described above. The extracted PCR products were resuspended in TE and the DNA concentration was determined by measuring absorbance at 260 nm in a UV spectrophotometer.

2.8.5 Amplicon digestion

In order to cleave the R adaptors both tester and driver amplicons were digested with the restriction endonuclease BamH I in 200 µl volume containing: BamH I (10 U /ug DNA), 1X buffer E, and 10µg of driver and tester amplicons for 2 hours at 37 C. Considering that a total of 150 µg of driver amplicons were produced, 15

independent digestions were required for each driver sample. In order to determine the success of the technique, 50 μg of tester amplicon was used as driver amplicon. The digested drivers amplicons were extracted as above. The R adaptors were removed from only the digested tester amplicons by Centricon-100 ultrafiltration (Amicon, Beverly, MA); 200 μl of tester digestion reaction was diluted in 2 ml of TE. The diluted tester was applied to the columns and centrifuged at 750 g for 30 min. The retained tester was diluted in TE to 100 ng/μl. The success of the digestion was checked by running 10 μl of ligated and digested reaction in a 2% agarose gel.

2.8.6 Ligation of new adaptors on tester amplicons

2μg of digested tester amplicons were ligated with new adaptors J24: ACCGACGTCGACTATCCATGAACG, and J12: GATCCGTTCATG, using the same ligation conditions used for the R24 and R12 adaptors. The success of the ligation was checked by agarose gel electrophoresis, where a 50 bp difference between digested and ligated fragment should be observed. The J ligated tester was diluted to 10 ng/μl with TE.

2.8.7 DNA hybridisation

All five driver amplicons were pooled and mixed by pipetting up and down several times. Forty micrograms of pooled digested driver amplicons and 0.4 μg of J ligated tester were combined. Forty micrograms of the tumour driver amplicon was also combined with 0.4 μg of J ligated tester. The driver-tester mixture was purified by Phenol/chloroform/isoamyl alcohol, and then precipitated by addition of 30μl of 10 M ammonium acetate (Sigma) and 2.5 volumes of ethanol, the amplicon mixture was placed on dry ice for 10 min followed by 2 min at 37 ^{0}C. The mixture was precipitated by centrifugation at 13,000 rpm for 15 min, and resuspended in 4 μl of 3 X EE buffer (30 mM EPPS, 3 mM EDTA pH 8.1), (the EPPS contained in the EE buffer was used because it does not acidify when heated to 98 ^{0}C), then incubated

for 5 min at 37 °C, and 30 µl of mineral oil was added. The amplicon mixture was denatured at 98 °C for 4 min in a heat block, and 1 µl of 5 M NaCl was added directly without removing the tube from the heat block, and mixed carefully to avoid the formation of bubbles. The mixture was then incubated for 24 hours at 67 °C.

2.8.8 Selective amplification

Once the mineral oil was removed, the hybridisation mixture was diluted in 400 µl of TE buffer. The hybridisation reaction was amplified in 400 µl volume containing: 1X PCR buffer, 0.4mM dNTPs, and 40 µl of hybridised mixture. The PCR reaction was incubated at 72 °C for 3 min, and then 20 U of Taq Polymerase was added for 5 min at 72 °C. To amplify the self-re-annealed tester DNA, 500 pmol of J24 primers were added. The first amplification consisted of 10 cycles at 95 °C 1 min and 70 °C 3 min) followed by 72 °C 5 min. The amplified products were extracted as above and resuspended in 40 µl of water, then treated with mung bean nuclease (New England Biolabs) to remove single strand DNA molecules. The reaction consisted of: 1 X mung bean nuclease buffer, 20 U of mung bean nuclease, 20 µl of amplified hybridisation products. The reaction was incubated at 30 °C for 30 min, the nuclease was inactivated by adding 160 µl of Tris-HCl pH 8.9 and incubated at 98 °C for 5 min. The mung bean nuclease treated product was purified by Centricon-100 filter as described above and diluted in 120 µl of water. 40 µl of the nuclease treated products were amplified using the J24 primers. Once the PCR reaction was overlaid with mineral oil and denatured at 95 °C for 1 minute, 20 U of Taq DNA polymerase was then added. The amplification conditions consisted of 20 cycles at 94 °C for 1 minute, followed by 70 °C for 3 minutes, with a final extension of 10 minutes at 72 °C. The PCR products were run on 1.5 % agarose gel to check the profile and yield.

2.8.9 Changing the adaptors for subsequent hybridisation/amplification step

The difference product (DP) from the first selective amplification was re-digested with BamH I to remove the J adaptors than ligated with new adaptors N24 and N12, following the same conditions as described above. The second and third rounds of hybridisation were repeated hybridising 50 ng and 100 pg of DP1 and DP2 respectively with 40 μg of drivers amplicon following the same conditions as described. Selective amplifications were repeated as described in the first selective amplification using the N24 and J24 oligonucleotides respectively.

After three round of hybridisation and selective amplification, the difference products were visualized by 1.5 % agarose gel electrophoresis.

2.8.10 Fourth hybridisation/amplification round

Given that the tester and drivers were genetically unrelated, in order to reduce the selective amplification of a fragment that represents a normal genetic polymorphism a fourth hybridisation was performed using pooled amplicons prepared as before from 10 unrelated mixed breed dogs. The hybridisation mixture containing 5 pg of DP3 and 40 μg of pooled drivers amplicon was hybridised and selectively amplified using the N24 primers. The amplified products were run along with a 100 bp ladder on 2% 1:1 NUSieve/agarose gel (FMC, Rockland, ME and Gibco/BRL) gel in 0.5 X TBE buffer and visualized by ethidium bromide staining.

2.8.11 Isolation and enrichment of difference products from NUSieve/agarose gel

Each visible band was excised from the gel with a different clean scalpel. In order to clone fragments that may not be sufficiently abundant to be seen, in addition to excising the visible bands, gel fragments in a range of 200-1500 bp, so that each fragment includes a 200-300 bp range, were excised. The excised gel fragments were resuspended in 200 μl for the more intense bands and 400 μl for the larger gel pieces, in Tris-HCL pH 8.9, and then melted for 7 min at 98 °C. Fourty μl volume of

diluted product extracted from larger gel pieces was re-amplified using the N24 primers using the same conditions as above with 15 amplification cycles. The purity of the re-amplified products was checked on 2% 1:1 NUSieve/agarose gel. The visible bands produced by re-amplification were excised as before and diluted in 200 µl of TE.

2.8.12 Cloning and screening of difference products

Purified PCR products from the fourth round were ligated into the pGEM-t Vector and transformed in super competent bacteria TOP 10 as described in section 2.12-2.13. Seven colonies from each cloned band were amplified by PCR using the N24 primers and purified using QIAGEN PCR Purification kit according to manual. In order to avoid cloning background material, purified products were digested with restriction endonuclease MnlI (New England Biolabs), which cuts frequently therefore provides a restriction fingerprint. The digested products were run on a 2% NUSieve gel. The fragment used as probe for southern blot analysis was selected by the criterion that if three or more of the seven colonies share a common restriction fingerprint, it was more likely to represent the difference product rather than background material.

2.9 Probe labelling

Selected inserts were labelled with alpha dATP 32 P by random primer labelling (HexaLabel DNA labelling Kit, Fermentas). The labelling reaction was carried out into a 1.5 ml microcentrifuge tube by adding the following components: 100 ng of DNA, 1 X reaction buffer mixed with hexanucleotides, and deionised water to 40 µl. The microcentrifuge tube was then vortexed and spun down for 5 seconds. The reaction was incubated in a boiling water bath for 10 minutes and cooled down on ice. Three µl Mix A (containing a mixture of dNTPs except dATP) and 500 µCi of α ^{32}P dATP (ICN) were added to the labelling reaction. In order to label the DNA

fragment with α^{32}P dATP, 5 units of Klenow fragment (exonuclease negative) was added. The reaction was then incubated at 37 °C for 15 minutes. Four µl (1mM) of dNTPs (including dATP) was added to the reaction and incubated for 5 minutes at 37 °C. The labelling reaction was stopped by adding 1 µl of 0.5 M EDTA (pH 8). The unincorporated dNTPs were removed by applying the labelling reaction in to pre-spin Sephadex G 50 columns and centrifuged at 3000 rpm for 2 minutes.

2.10 Southern blots

0.5 µg of driver and tester amplicons from the first amplification were subjected to electrophoresis on 2% NUSieve agarose (FMC Byproducts) for 2 hours at 60 volts. The DNA was visualised by staining the gel with ethidium bromide (0.5 µg/ml). In order to denature the DNA amplicons, the gel was transferred to a Pyrex baking dish containing 0.4 M of sodium hydroxide and 1.5 M of sodium chloride, for 10 minutes on a rotary shaker. The gel was then neutralised with a solution containing 1.0 M of Tris-HCl pH 8.0, 1.5 M of sodium chloride and then with deionised water. To transfer the DNA amplicons a positively charged nylon membrane Hybond-Xl was used (Amersham Pharmacia, Biotech). The membrane was cut 2 mm larger than the gel in both directions and then washed in deionised water and briefly in 0.4 M of NaOH. The DNA was transferred overnight by capillary transfer to a membrane using 0.4 M of sodium hydroxide as transfer buffer.

2.10.1 Hybridisation

After transfer the membrane was removed from the gel and placed in a tray containing 2X SSC (Sodium c hloride Sodium Citrate) (20X SSC: 3M Sodium chloride, 0.2 Sodium citrate; pH 7.0) and 0.5 % sodium dodecilsulphate (SDS) for 15 minutes. The DNA was cross-linked to the membrane using the UV-linker Stratalinker (Stratagene). The membrane was transfered to a Pyrex hybridisation tube containing 10 ml of Hyb solution (Amersham) and 10 µg/ml of salmon sperm DNA, and incubated in a roller hybridisation oven at 65°C for 30 minutes. After the

pre-hybridisation, a mixture containing 50 µl of labelled probe and 1ml of Hyb Solution was heated in a boiling water bath for 7 minutes and immediately transferred to the hybridisation tube. Hybridisation was performed at 50 °C for 24 hours.

2.10.2 Washing and detection.

After the removal of the hybridisation solution, a solution containing 150 ml of 2X SSC, and 0.1 % of sodium dodecilsulphate (SDS) was added to the tube and incubated for 15 minutes in the roller hybridisation oven. This procedure was repeated twice more but using 1X SSC the first time and 0.1 X SSC the second time. After the membrane was dried it was exposed to the Phospho Imager cassette for 24 hours and analysed using the Storm 860 scanner (Molecular Dynamics).

2.11 Non- denaturing polyacrylamide gel electrophoresis

Cy5 labelled PCR products were run using 5% polyacrylamide gel containing: 1x TBE (90 mM Tris-borate, 2 mM EDTA pH 8.3) 5% Long Ranger (Cambrex) (containing polyacrylamide) and distilled water up to 50 ml. To polymerise 25 µl of N, N, N', N' Tetramethylethylenediamine (TEMED), and 250 µl of 10% ammonium persulfate were added and mixed for 2 minutes. After polymerisation the gel was run in 1X TBE buffer at 50 V for 1-2 hours. PCR products were detected using the Storm 860 phosphoimager (Molecular Dynamics)

2.12 Ligation of PCR product into cloning vector

Gel purified PCR products were ligated in to pGEM-T vector (Promega). This linear vector possesses overhanging thymidines at both 3' ends, therefore enabling the ligation of PCR products that have been adenosylinated at the 3'ends by Taq DNA polymerase. The ligation reactions were carried out in a total volume of 10 µl containing: 5 µl of 2X rapid ligation buffer, 1µl of T4 DNA ligase (3 units), 1 µl of Vector (50 ng), and 150 ng of PCR product; in all cloning procedures an insert:vector ratio of 3:1 was used, the volume was adjusted to 10 µl with deionised

water. All components were mixed and centrifuged for 3 seconds at 13,000 rpm. The reaction was incubated overnight at 4 ^{0}C.

2.13 Transformation of chemically competent bacterial cells

Transformation was carried out using chemically competent One Shot TOP10 cells (Invitrogen), which are compatible with blue/white screening and ampicillin selection. Competent cells (50 µl) contained in a 1.5 ml vial were thawed on ice and 2 µl of the ligation reaction was added directly into the competent cell solution and mixed by gently tapping. The cells were then incubated for 30 minutes on ice. Cells were heat shocked at 42 ^{0}C for 30 seconds and then placed on ice for 2 minutes. 250 µl of pre-warmed SOC medium was added to the transformed cells and incubated at 37 ^{0}C for 1 hour in a shaking incubator at 225 rpm. 100 µl of transformed culture were spread in on LB agar plates containing 100µg/ml ampicillin, 0.5 mM of ispropyl-β-D-thiogalactopyranoside (IPTG) (Promega) and 80 µg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X Gal) (Promega). The LB plates were then incubated overnight at 37 ^{0}C.

2.14 Screening transformants for insertion

The pGEM-T vector contains the cloning site within the β-galactosidase gene, which encodes for an enzyme that converts the colourless X Gal substrate into a blue dye. Thus successful ligation interrupts the β-Gal coding sequences and colonies with the insert are white, and colonies without an insert are blue. Positive white colonies were subsequently analysed by PCR.

2.15 Isolation and purification of plasmid DNA

Selected white colonies were inoculated into 3ml of LB medium containing 100 µg/ml ampicillin, and then incubated overnight at 37 ^{0}C in a shaking incubator at 250 rpm. The next day the culture was centrifuged at 10,000 rpm for 1 minute, and after the removal of the supernatant the plasmid DNA was extracted using the QIA

miniprep kit (QIAGEN); the pellet was resuspended in 250 µl of Buffer P1, the bacterial cells were lysed by adding 250 µl of Buffer P2 (alkaline buffer) and after inverting the microcentrifuge tube 5-6 times, and the mixture was incubated for 5 minutes. The lysate was neutralised by adding 350 µl of buffer N3 and inverting the tube 5-6 times. To separate plasmid DNA from genomic DNA and proteins, the lysate was centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a QIAGEN mini column, which contains an anion exchange resin able to bind DNA. The plasmid DNA was isolated by aspirating the supernatant through the column using a vacuum system. The DNA bound to the column was then washed by adding 750 µl of buffer AW and centrifuged 2 times at 13,000 rpm for 1 minute to remove residual buffers and to dry the column. Plasmid DNA was eluted by adding 50 µl of deionised water in the centre of the silica gel membrane and centrifuged at 13,000 rpm for 1 minute. The DNA concentration was determined using Nanodrop.

2.16 Automated DNA sequencing

The insert cloned in to pGEM-t Vector was sequenced by adding 150 ng of purified plasmid in a sequencing reaction containing: 3.2 pmol of vector or insert specific primers and 8 µl of Dye Terminator Cycle Sequencing mix (Beckman Coulter) and sterile deionised water up to a total volume of 20 µl. The reaction components were mixed thoroughly and then centrifuged at 13,000 for 2 seconds. Amplification was performed on an MJ research PTC 200 thermal cycler with a heated lid using the following conditions: an initial denaturation at 92 ^{0}C for 2 minutes, followed by 30 cycles consisting of 20 seconds at 96^{0}C, 20 seconds at 50 ^{0}C and 4 minutes at 60^{0}C. To precipitate the amplified product, the sequencing reaction was transferred to a sterile 0.5 ml microcentrifuge tube containing 4 µl of stop solution freshly prepared by mixing equal volumes of 3M sodium acetate pH 5.2 and 100 mM ethylenediaminetetraacetic acid (EDTA) and 1 µl of 20 mg/ml of glycogen. After mixing thoroughly, 60 µl of cold (-20 ^{0}C) 95% ethanol was added and mixed by pipetting up and down several times. The tube was immediately centrifuged at

14,000 rpm at 4 ^{0}C for 30 minutes. After centrifugation the supernatant was carefully removed and the pellet was washed twice with 200 µl cold (-20 ^{0}C) 75% ethanol, each time the tube was centrifuged at 14,000 rpm at 4 ^{0}C for 3 minutes. After removal of all supernatant, the pellet was vacuum dried at room temperature for 40 minutes and then resuspended in 40 µl sample loading solution (Beckman Coulter) containing deionised formamide. The resuspended product was then transferred into 96 well plates and overlaid with a drop of mineral oil. The sequencing product was run in a Beckman CEQ 2000 DNA analysis machine.

2.17 Genetic and phylogenetic analysis

In order to analyse the genetic variation, DNA sequences were aligned by using BioEdit Sequence Alignment Editor 5.0.9 (Hall, 1999). To analyse the genetic relationship between tumour and normal sequences, DLA and mtDNA sequences available from GenBank were also analysed. A Blast analysis was also performed to see if tumour and normal sequences found in this study had been already described. Phylogenetic analyses were made using PAUP version 4.0 beta version (Swofford, 2001). The mtDNA neighbor joining tree was constructed according to the LogDet model. The LogDet method assumes that all sites of a specific sequence can vary at the same rates, and allows the base frequency or base composition to vary over the tree (Tamura and Kumar, 2002). Unlike other methods that assume complex but specific models of nucleotide substitution, the LogDet model was chosen in order to correct for potential multiple substitutions (otherwise the evolutionary distances would be under-estimated) and could be used without collapsing the topology of the tree.

The substitution model to construct the trees was chosen using Model Test 3.06 (http://inbio.byu.edu/Faculty/kcac/Crandall_lab/modeltest.html). Model Test (Posada and Crandall, 1998) compares different models of nucleotide substitution, calculating the likelihood ratio test (LTR) scores and Akaike Information Criterion (AIC) values for each of the 50 models. The output file containing the best-fit model

selected by the program is then used by the PAUP program to compute the phylogenetic tree (neighbor joining, maximum parsimony, maximum likelihood). For MP or ML methods the best tree is computed by a heuristic search, whereas for NJ and MP methods bootstrap analysis can be performed. For the DRB1 a ML phylogenetic tree was built using a K81uf model (GTR+G+I) (selected by AIC in Model Test), and the best tree was chosen from 333,025 simulated trees (with a running time of 16 hours). The genetic relationship between tumour, host sequence and sequence present in the mtDNA Clade A, was also analysed by using a median joining networks calculated by Network 4.0 (http://www.fluxus-engineering.com) (Bandelt et al., 1999). The Median Joining method consists of two algorithms. The first finds the minimum spanning trees for a set of sequences, without creating any connection between them and without inferring additional node; the second algorithm elaborates maximum parsimony trees by creating a median vector between sequences. Median vectors can be interpreted as consensus sequences, which represent unsampled sequences or extinct ancestral sequences. A default setting of $\varepsilon = 0$ and with identical weight between nucleotide was chosen to elaborate the MJ network.

2.18 Microsatellite statistical analysis

This analysis was undertaken in collaboration with Jonathan Pritchard at the University of Chicago. In order to analyse the genetic relationship between tumour and its host, and between tumours, a Bayesian clustering model implemented in the program Structure was used (Pritchard et al., 2000). The Structure algorithm constructs genetic clusters using microsatellite frequency data to infer population structure and to assign probabilistically individuals to populations. This method does not assume any particular mutational process, but assumes that markers within populations are in Hardy-Weinberg equilibrium. The program adopts a Bayesian inference model to estimate several prior parameters such as: allele frequencies in all population, population of origin of the individual and the proportion of the individual genome that originated from a specific population. The essence of the Bayesian method is to infer the posterior probability using the joint distribution

between prior parameters, distribution and likelihood distribution according to the following formula:

$$p(\Phi|X) = \frac{p(\Phi)\, p(X|\Phi)}{p(X)}$$

where $p(\Phi|X)$ represents the posterior distribution of the parameters given the data $p(\Phi)$ is the prior distribution of the parameters , $p(X|\Phi)$ is the likelihood that specify the probability of the data given any particular parameters values and $p(X)$ prior represents the distribution of the data (Shoemaker et al., 1999; Beaumont and Rannala, 2004). The join distribution was estimated using a Markov Chain Monte Carlo method.

In this study 11 tumours and 11 host genotypes consisting of 5 dinucleotide and 4 tetranucleotide markers were run. First I performed 10 independent runs with 100,000 iterations following a burn-in period of 10,000 iterations, assuming prior K values (number of clusters) from 1 to 5 and without prior information regarding the origin of the tumour. An ancestry model with admixture (where the individuals may have mixed ancestry) and uncorrelated allele frequency (where the allele frequencies in each population are independent) was first performed. Second sets of runs were performed using an unmixed model (where each individual comes purely from one specific population) and correlated frequency (allele frequencies in different population are similar).

In order to determine the origin of CTVT, a tumour consensus genotype based on 73 dinucleotide markers was compared with normal genotype obtained from 414 pure-bred dogs representing 85 breeds (five unrelated dogs for each breed) and from 8 unrelated wolves (from China, Oman, Iran, Italy, Sweden, Mexico, Canada, and United States). 20 independent runs using 100,000 iterations and a burn in period of 20,000 iterations with admixed and uncorrelated models and with K=4 were performed without prior origin (breed) information. Following the determination of

the cluster in which the tumour was assigned, all individuals within the cluster were used to assign the tumour to a specific population. Two data sets were used: in the first data set dogs and wolves were analysed together with the tumour genotype, whereas in the second data set only the tumours and dogs genotype were analysed. The runs consisted of 100,000 + 20,000 iterations with admixed, uncorrelated or correlated models. For dogs and wolves, prior origin information was used but no prior origin information was used for the tumours. Microsatellite genetic distances were estimated by two methods: chord distance (Cavalli-Sforza and Edwards, 1967), and proportional shared allele (Dps) distance (Bowcock et al., 1994) using MICROSAT(http: hpgl.stanford.edu projects microsat.htlm) (Goldstein et al., 1995). NJ phylogenetic trees were constructed with the programs Neighbor and Consense in the PHYLIP3.5.(http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1989).

CHAPTER 3

Search for an aetiological agent by RDA

3.1 Introduction

Cancer can be viewed as a genetic disease, where the heritable genetic alterations are somatically transmitted (Nowell, 1976). The genetic alteration that initiates the transformation can be caused by chemical and physical agents or by infectious agents. Among infectious agents viruses have been implicated as causative agents in several tumours in animal and humans (Gross, 1983; Wyke and Weiss, 1984).

The reproducibility of CTVT either naturally by coitus or experimentally by inoculation of tumour cells, led scientists to investigate whether an infectious agent induced the tumour (Gross, 1983). The search for an oncogenic virus was attempted either by inoculation of tumour cell free extract or by electron microscopy. Although some investigators (Ajello, 1960; Dozza and Torlone, 1960b) reported the successful transmission of the tumour by lyophilised cell extract inoculations, numerous attempts to reproduce the disease failed to transmit it by tumour cell free extracts. However, the presence of crystalline virus-like particle structures has been observed in experimentally reproduced tumours (Ajello and Gimbo, 1965; Lombard and Cabanie, 1967; Kennedy et al., 1977).

Despite of numerous efforts so far, all results have failed to identify an infectious agent able to induce and transmit the tumour. However it has been suggested that an infectious agent(s) may have originally induced CTVT, but that viral involvement is no longer be required for the progression and transmission of the tumour (Kennedy et al., 1977). It is also possible that a defective virus might play a synergistic role with the tumour cell during the pathogenesis of the tumour.

Wigler and Lisitsyn (Lisitsyn et al., 1993), developed a powerful and versatile gene discovery method called Representational Difference Analysis (RDA) which allow the analysis of the differences between any two sets of DNA, such as normal versus tumor DNA, or uninfected cells versus cells infected with an unknown virus. Recently RDA has been used to search for foreign sequences belonging to an infectious agent in AIDS-associated Kaposi's Sarcoma, Multiple Sclerosis and GB hepatitis (Chang et al., 1994; Challoner et al., 1995; Simons et al., 1995). RDA analysis led to the identification of a new Human Herpesvirus (HHV8) as the causative agent of the Kaposi's sarcoma. Interestingly, before the identification of the HHV8, epidemiological studies suggested that like CTVT, Kaposi's sarcoma might be caused by cellular transmission (Hayes et al., 1983). Therefore conversely, the finding that Kaposi's sarcoma is virus-induced suggests that an unknown virus may also be the causative agent of CTVT.

In addition, RDA has been used in tumours to detect genetic lesions including rearrangement, amplifications and deletions (Lisitsyn et al., 1995). It is also been used to obtain polymorphic DNA markers for genetic analysis in several species including dogs (Lisitsyn et al., 1994; Everts et al., 2000). Although the abnormal karyotype of CTVT was described a long time ago, so far no detailed genetic studies have been undertaken. Therefore given that RDA allows the detection of genetic differences between normal and tumour cells, in this study I have utilised the RDA method to seek to identify DNA of a pathogen or to detect host genetic abnormalities associated with CTVT. (See Fig 2.3 in Chapter 2 for the schematic protocol of the representational difference analysis).

3.2 Results.

In order to avoid DNA contamination of external pathogens or microbes, RDA was performed with tissue from an internal metastatic tumour as tester DNA and a pool of normal DNA from different unrelated dogs as drivers. The driver amplicons were

produced independently and mixed before hybridisation at the fourth step. To test the success of the first round of subtraction, individual driver amplicons were tested with the tester amplicon. Fig 3.1 shows the Difference Products (DP) of the first round, where several bands of different sizes from 500 to 1300 bp are present when drivers and tester were hybridised, while hybridising the tester amplicons to each other do not produce any visible band. After three rounds of hybridisation several bands were still present in all driver samples (Fig 3.2).

Given that the tumour is genetically not related with the host, to subtract the normal polymorphic sequences and the sequence repeats present in the different products after the third round (Fig 3.2), a second pool of driver amplicons from ten unrelated dogs was used in the fourth round. Fig 3.3 shows the difference products from the first to the fourth round. After four rounds of RDA a total of 9 bands was obtained. In order to differentiate the DNA fragments having the same size but different sequences, the PCR products were digested with MnlI. From these bands, after MnlI fingerprint selection 10 DP were isolated. All 10 DP products were labelled and analysed by Southern blot. Of 10 selected probes, 8 showed hybridisation only with the tester amplicon (Fig 3.4) whereas clones 40 and 13 hybridised with all driver amplicons.

After sequencing the DP products, clone 37 was found to be identical to clone 39. In order to search for sequence homology, all DP sequences were analysed by Basic Local Alignment Search Tool (BLAST). None of the sequences showed any similarity with pathogen sequences. Although the sequences do not match canine gene sequences, they show more than 90% similarity to canine genome non-coding sequences located in different chromosomes.

To see if the DP were close to genes, sequences were run against the recently published dog genome using Ensembl software. Fig 3.5 shows the physical assignment of the DPs in different chromosomes.

Although no pathogens or cancer-related genetic alterations have been found, the success of the RDA technique was demonstrated by the detection of polymorphic markers associated with CTVT.

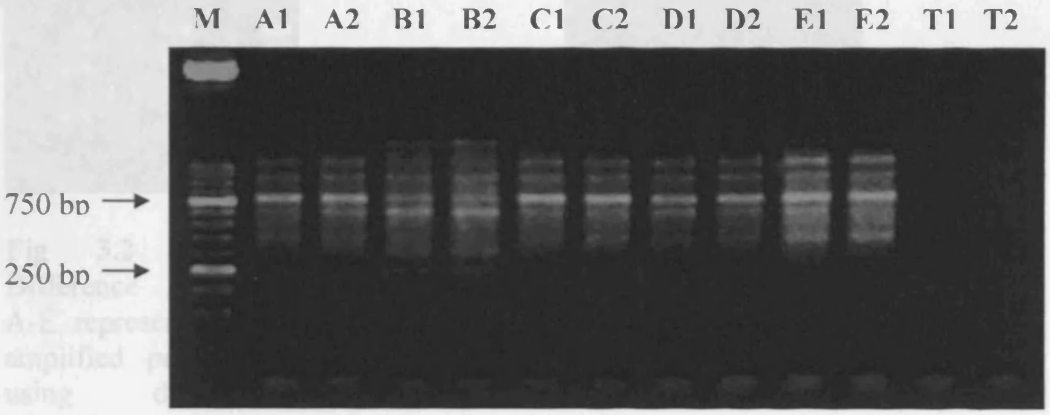


Fig 3.1. First round of hybridization/amplification . For each amplicons 2 tubes were yielded; lanes from A1 to E2, represent different products 1 (DP1) obtained hybridizing each driver amplicon against tester amplicon; Lanes T1-T2, represent the DP1 hybridizing tester samples each other. Lane 1 (M) contains the 50 bp DNA ladder.

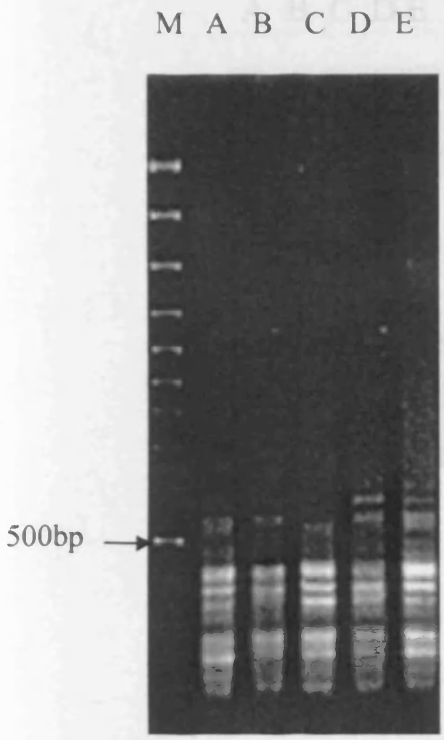


Fig 3.2 Third round Difference Products. Lane A-E represent the selective amplified product obtained using drivers A-E respectively. Lane 1 (M) contains 100 bp DNA ladder

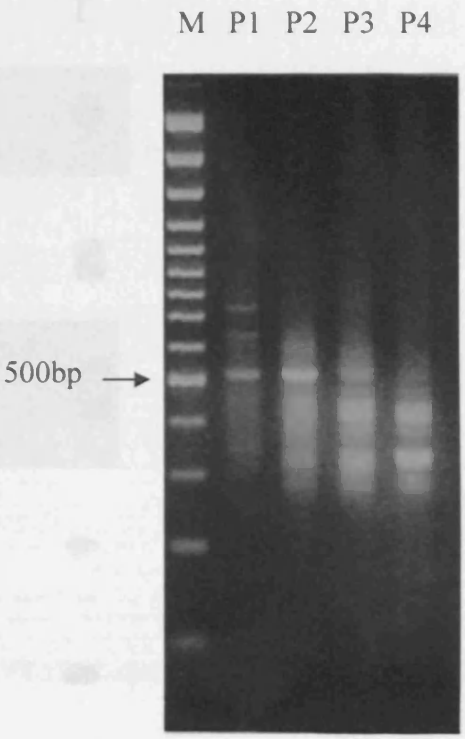


Fig 3.3 RDA Difference Products. Lanes P1 to P4, contain the first, second, third, fourth round DP products respectively. Lane 1 (M) contains 100 bp DNA ladder

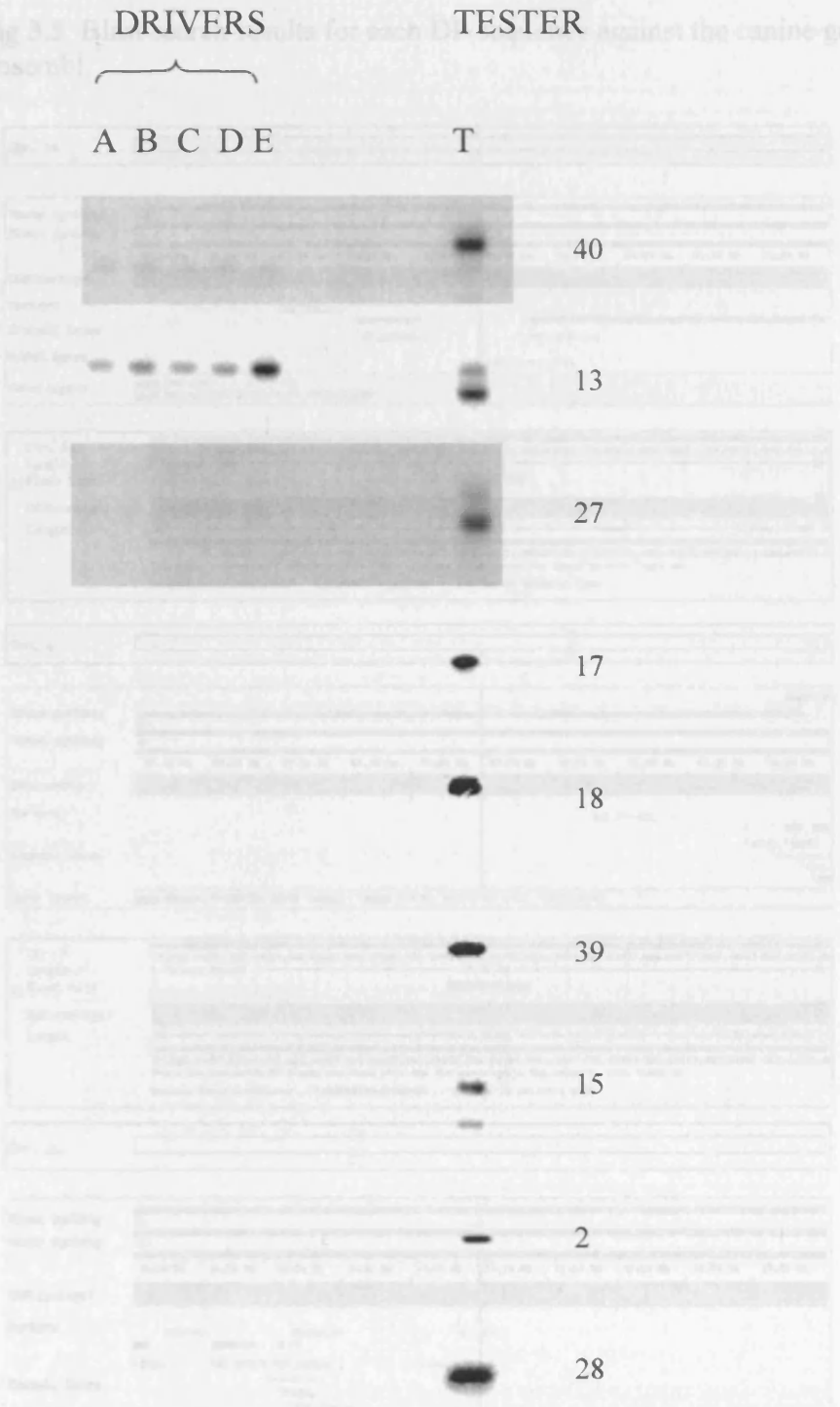
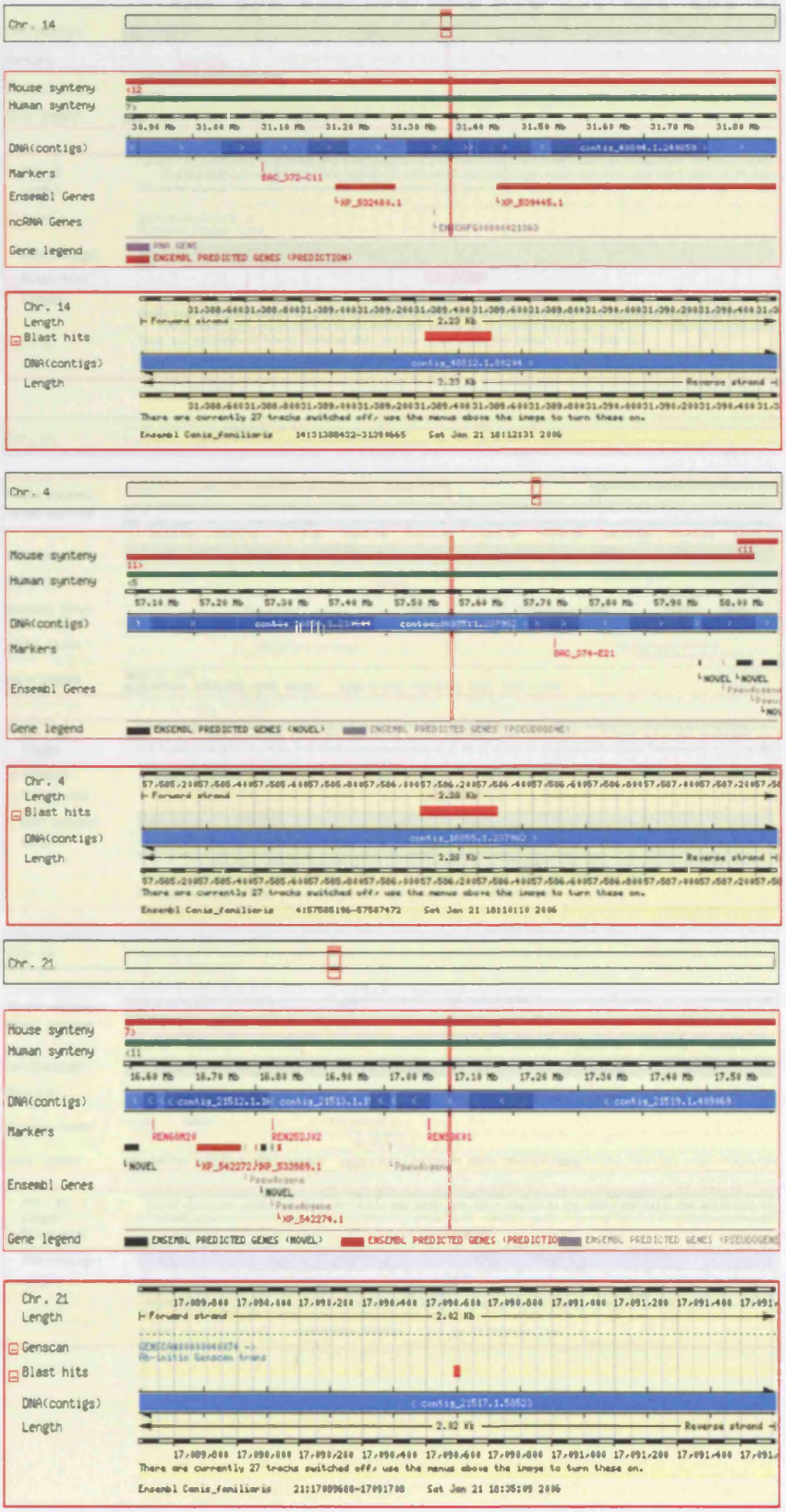


Fig 3.4 Southern blotting of the RDA amplicons derived from BamHI – representation DNAs from normal and tumour samples, hybridized with probes representing the differential products. Lane T, indicates tester amplicons; Lanes A, B, C, D and E indicate drivers amplicons.

Fig 3.5 Blast search results for each DP sequence against the canine genome using Ensembl.



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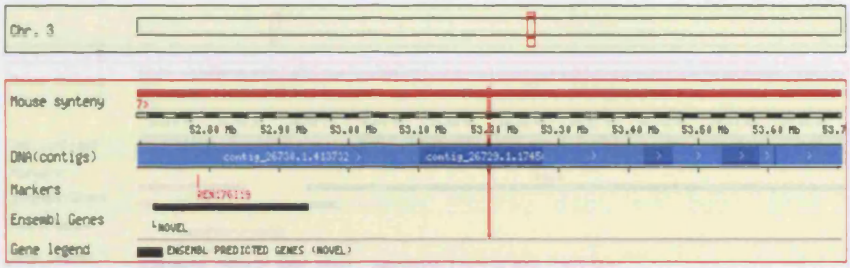
13

27

103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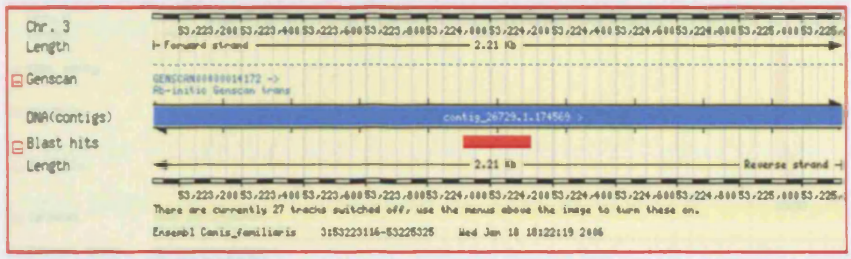
DNA(contigs)
Markers
Ensembl Genes
Gene legend
NOVEL
ENSEMBL PREDICTED GENES (NOVEL)

17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Length
Forward strand
2.21 Kb
Genscan
DNA(contigs)
Blast hits
Length
Reverse strand
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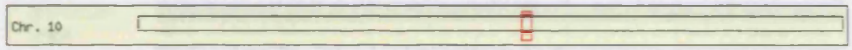
Ensembl Canis_familiaris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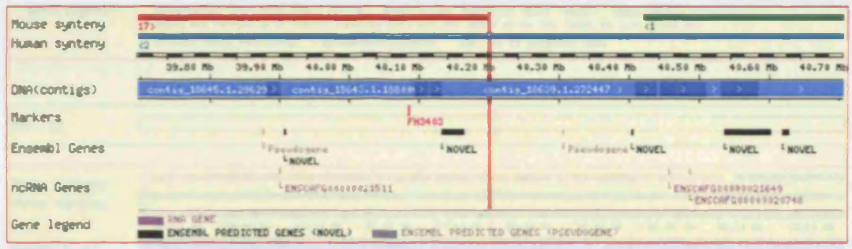
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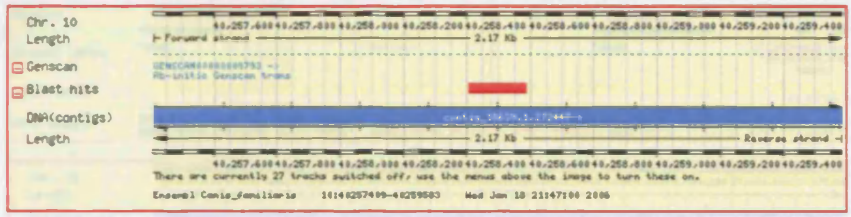
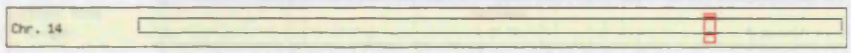
Human synteny
DNA(contigs)
Markers
Ensembl Genes
ncRNA Genes
Gene legend
NOVEL
ENSEMBL PREDICTED GENES (NOVEL)

18


Chr. 10
Length
Forward strand
2.17 Kb
Genscan
Blast hits
DNA(contigs)
Length
Reverse strand
There are currently 27 tracks switched off; use the menus above the image to turn these on.
Ensembl Canis_familiaris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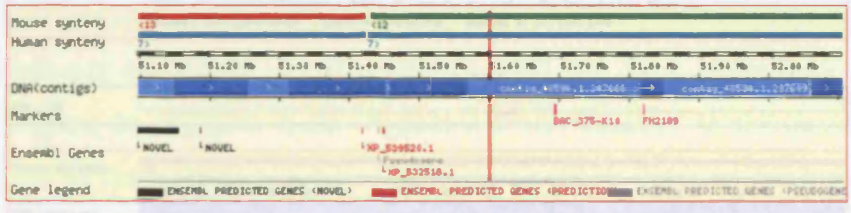
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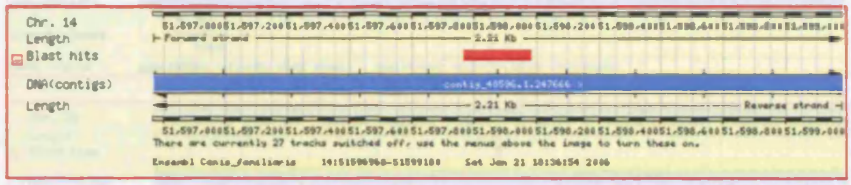
Human synteny
DNA(contigs)
Markers
Ensembl Genes
Gene legend
NOVEL
ENSEMBL PREDICTED GENES (NOVEL)

39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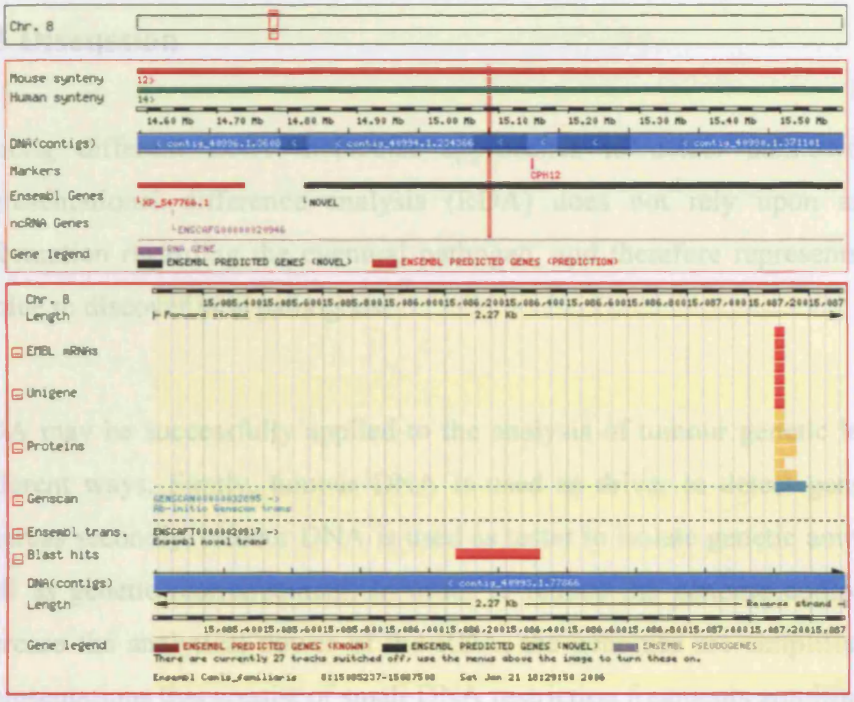
Length
Forward strand
2.21 Kb
Blast hits
DNA(contigs)
Length
Reverse strand
There are currently 27 tracks switched off; use the menus above the image to turn these on.
Ensembl Canis_familiaris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Human synteny
DNA(contigs)
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Ensembl Genes
ncRNA Genes
Gene legend
ENSEMBL PREDICTED GENES (NOVEL)
ENSEMBL PREDICTED GENES (PREDICTION)
Chr. 8 Length
EMBL mRNAs
Unigene
Proteins
Genscan
Ensembl trans.
Blast hits
DNA(contigs)
Length
ENSEMBL PREDICTED GENES (KNOWN)
ENSEMBL PREDICTED GENES (NOVEL)
ENSEMBL PSEUDOGENES
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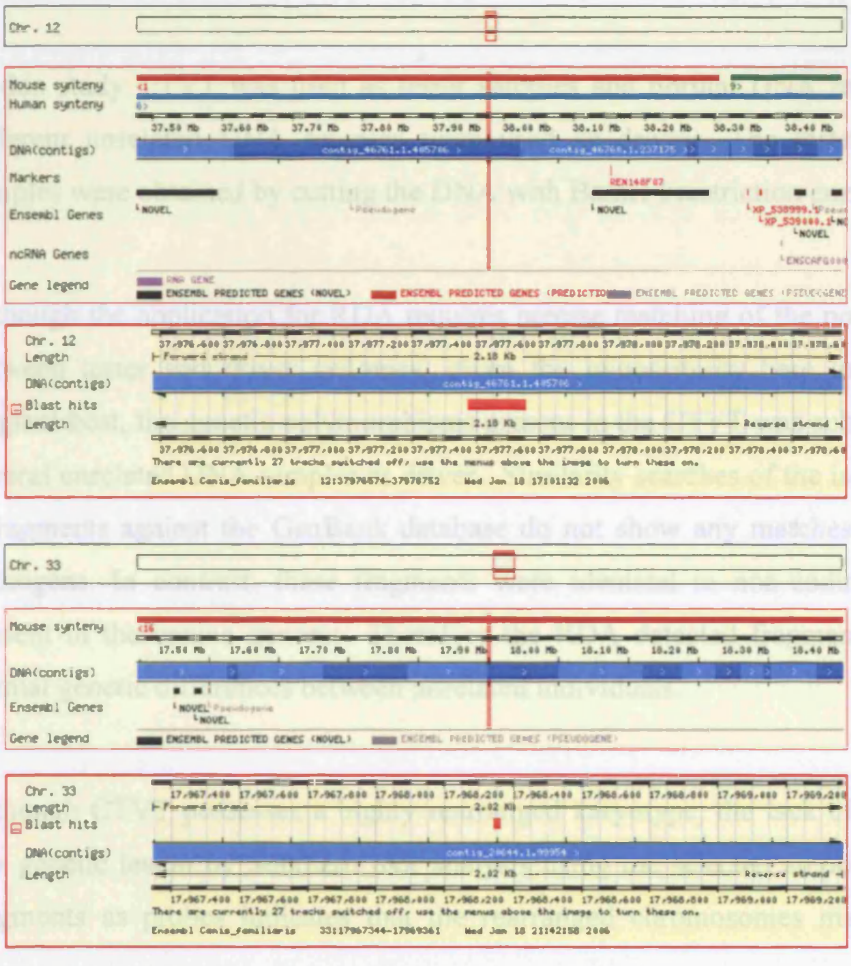
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Human synteny
DNA(contigs)
Markers
Ensembl Genes
ncRNA Genes
Gene legend
ENSEMBL PREDICTED GENES (NOVEL)
ENSEMBL PREDICTED GENES (PREDICTION)
Chr. 12 Length
Blast hits
There are currently 27 tracks switched off, use the menus above the image to turn these on.
Chr. 33
Mouse synteny
DNA(contigs)
Ensembl Genes
Gene legend
ENSEMBL PREDICTED GENES (NOVEL)
Chr. 33 Length
Blast hits
There are currently 27 tracks switched off, use the menus above the image to turn these on.

2

28

3.3 Discussion

Among different DNA molecular approaches to detect unknown pathogens, representational difference analysis (RDA) does not rely upon any sequence information regarding the eventual pathogen, and therefore represents the optimal choice to discover new pathogens.

RDA may be successfully applied to the analysis of tumour genetic lesions in two different ways. Firstly, tumour DNA is used as driver to detect genetic deletion, whereas secondly, tumour DNA is used as tester to isolate genetic amplifications as well as genetic rearrangement. In order to reduce the genome complexity and to increase the analytical power of the RDA, the genomes are simplified by making representations that consist of small DNA restriction fragments amplified by PCR.

In this study CTVT was used as tester samples and normal DNA represented by different unrelated DNA samples were used as drivers. The tester and driver samples were obtained by cutting the DNA with BamH I restriction enzyme.

Although the application for RDA requires precise matching of the polymorphisms between tester and driver genomes, given the impossibility here to analyse the original host, the genetic polymorphisms present in the CTVT was subtracted using several unrelated DNA samples as driver. Similarity searches of the isolated BamH I fragments against the GenBank database do not show any matches with known pathogens. In contrast, these fragments were identical to non-coding sequences present in the canine genome. Therefore the RDA detected fragments represente normal genetic differences between unrelated individuals.

Although CTVT possesses a highly rearranged karyotype, the lack of detection of any genetic lesion by Southern blot analysis using the selected amplified BamH I fragments as probes indicates that the rearranged chromosomes may be due to

chromosome fusion rather than chromosome rearrangements. However it should be pointed out that the unsuccessful detection of genetic lesions or foreign pathogens sequences in CTVT may be due to the fact that the RDA search was limited by the use of only one enzyme BamH I to isolate the genetic differences. Furthermore the search for a pathogen was limited to DNA pathogens, thus missing RNA or single-stranded DNA viruses.

CHAPTER 4

Clonal identity of tumour

4.1 Introduction

Cancer develops through an evolutionary process, where the neoplastic cell carrying the most advantageous mutated genome is selected and undergoes clonal expansion (Nowell, 1976; Greaves, 2002). The involvement of the selection process implies genetic diversity between tumour cells. The genetic diversity in cancer is generated through a process called genetic instability. Thus during tumour progression, genetic instability increases genetic divergence within the tumour, whereas natural selection minimizes tumour genetic diversity (Nowak et al., 2005). Cytogenetic and X inactivation analysis suggested that tumours are monoclonal in origin (Fialkow, 1979). Recently genotyping analyses using microsatellite markers have confirmed the monoclonal origin of several tumours (Fujii et al., 2000; Van Tilborg et al., 2000). Recent results have suggested that during clonal expansion tumour cells form a patch (genetic island), where tumour cells are genetically homogeneous (Garcia et al., 2000; Braakhuis et al., 2003). During tumour progression as the lesion becomes larger, clonal divergence gives rise to various sub-clones that form their own patches. Tumours are considered dynamic ecosystems, in which the genotype and habitat size of the tumour cell populations change through time (Tomlinson, 1997; Gonzalez-Garcia et al., 2002). While early or founder mutations are expected to be shared by all tumour cells, later mutations might be restricted to sub-clones and therefore to a specific topographical areas. Given these observations, the assessment of tumour clonality is constrained to the time of the analysis. Thus several genetic markers and multiple sampling are needed to determine tumour clonality (Garcia et al., 2000).

In the 1960s it was postulated from cytogenetic studies that CTVT might have originated from a single tumour cell clone (Takayama and Makino, 1961).

Additional genetic studies have shown the presence of a Long Interspersed Element (LINE-1) insertion upstream of c-*myc* gene, in tumours from several countries thus supporting the monoclonal origin of CTVT (Katzir et al., 1987; Amariglio et al., 1991). However these studies did not analyse paired normal and tumour tissue obtained from the same animal bearing natural occurring tumours. Although this LINE–1 insertion might represent a somatic rearrangement acquired during tumour progression, it might alternatively represent an inherited genetic marker present in a particular breed or in a family (Brooks et al., 2003).

This chapter describes the genetic analysis of naturally occurring tumours and their hosts collected from 1976 to 2005 in 5 different continents. The genetic analysis was based on several genetic polymorphic markers, including four major histocompatibility (MHC) genes, microsatellites, and mitochondrial (mt) DNA. The Line-1/c-*myc* rearrangement was also used as marker to test the presence of this insertion in naturally occurring tumours. These genetic markers were used to determine the genetic distance between tumours and their hosts and the genetic divergence between tumours from different geographical origins.

4.2 Results

4.2.1 Line –1/c-*myc* rearrangement tumour-host DLA haplotype

Using specific primers flanking the 3' insertion site to amplify the Line-1/c-cmyc rearrangement, we found that all tumours but none of the matched normal samples possessed this LINE-1 insertion (Fig 4.1 a-b). This finding indicated that the tumours did not require a host germline LINE-1 insertion, and that the LINE-1 insertion site can be used as diagnostic marker of the tumour. A further 21 paraffin embedded tumours were found to carry the insertion (Fig 4.1b) consistent with previous studies (Katzir et al., 1987; Liao et al., 2003).

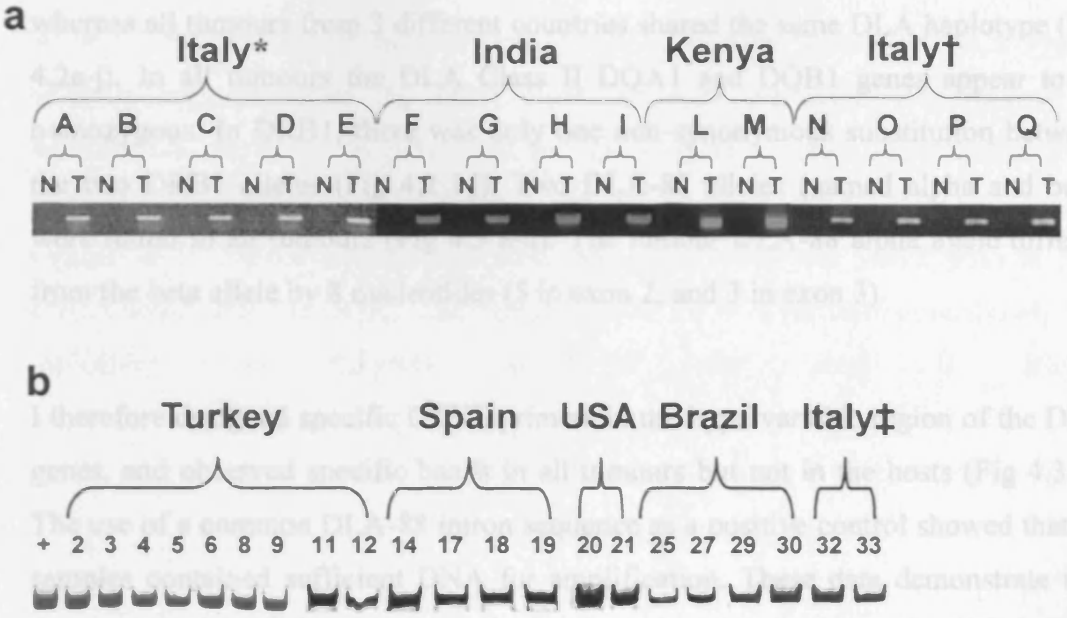


Fig 4.1 LINE-1/c-*myc* PCR amplification. **A**: PCR amplification from fresh tumours. For each dog (A to Q) fresh normal and tumour host samples are indicated with N or T. **b**: PCR amplification of microdissected tumour cells from paraffin-embedded specimens. * = Catania, † = Messina, ‡ = Sardinia

4.2.2 CTVT-host DLA haplotype

In order to determine the genetic diversity between tumour and host and the genetic divergence between tumours of the same or different geographic areas, the most polymorphic genes of the canine MHC (also known as Dog Leukocyte Antigens DLA) one class I (DLA-88) and three class II (DRB, DQB1, DQA1) were analysed (Fig 4.2 a-j). Given that the greatest genetic variation is present in exons 2 and 3 of the DLA-88 and in exon 2 of the DLA class II genes, these regions were amplified using flanking intronic primers.

Given that tumour tissue contains normal stromal cells, the tumour specific DLA haplotype was determined to eliminate the alleles found in the paired host.

In each dog examined the CTVT DLA haplotype was different from the host, whereas all tumours from 3 different countries shared the same DLA haplotype (Fig 4.2a-j). In all tumours the DLA Class II DQA1 and DQB1 genes appear to be homozygous. In DRB1, there was only one non-synonymous substitution between the two DRB1 alleles (Fig 4.2 i-j). Two DLA-88 alleles (named alpha and beta) were found in all tumours (Fig 4.3 a-d). The tumour DLA-88 alpha allele differed from the beta allele by 8 nucleotides (5 in exon 2, and 3 in exon 3).

I therefore designed specific CTVT primers in the hypervariable region of the DLA genes, and observed specific bands in all tumours but not in the hosts (Fig 4.3 a). The use of a common DLA-88 intron sequence as a positive control showed that all samples contained sufficient DNA for amplification. These data demonstrate that the tumours from Italy, India, and Kenya share the same haplotype in all four DLA genes

The DLA typing analysis was extended to microdissected tumour tissue derived from paraffin embedded specimens obtained from Brazil, USA, Turkey, Spain and Italy. Given that the DNA extracted from fixed samples is degraded and that the length of the PCR product obtained using the tumour specific DLA-88 primers was too long (450 bp), only the class II genes amplicons (150 bp) were analysed in microdissected tumour samples. The tumour specific DLA DRB1, DQB1 and DQA1 alleles were shared by all tumours analysed (Fig 4.3 b).

In order to see if the tumour specific alleles have been previously described, a comparison between the CTVT DLA sequences and the sequences present in GenBank was performed. The DLA-88 and DRB1 alleles found in the tumours have not been identified so far in any canine species; however, the CTVT DQB1 and DQA1 alleles have been described, DQB1 03501 in North American and European wolves and in dogs (mongrels and Shih Tzu), and DQA1 04202 only in Huskies.
The possible breed of origin is analyzed further in Chapter 7.

Although the tumour is heterozygous for the DLA-88 locus, the DLA typing analysis suggested that the DLA class II genes are either homozygous or hemizygous. In order to determine the DLA genetic status, quantitative PCR analysis was performed (Fig 4.4). While the DLA-88, DRA, DRB1 loci were diploid in all tumour samples analysed, the DQB1 locus was hemizygous in 5/11 fresh tumours and the DQA1 was hemizygous in 12/19 of the tumour analysed, thus indicating the frequent loss of the class II alleles.

Fig 4.2a. DLA-88 exon 2 nucleotide alignment

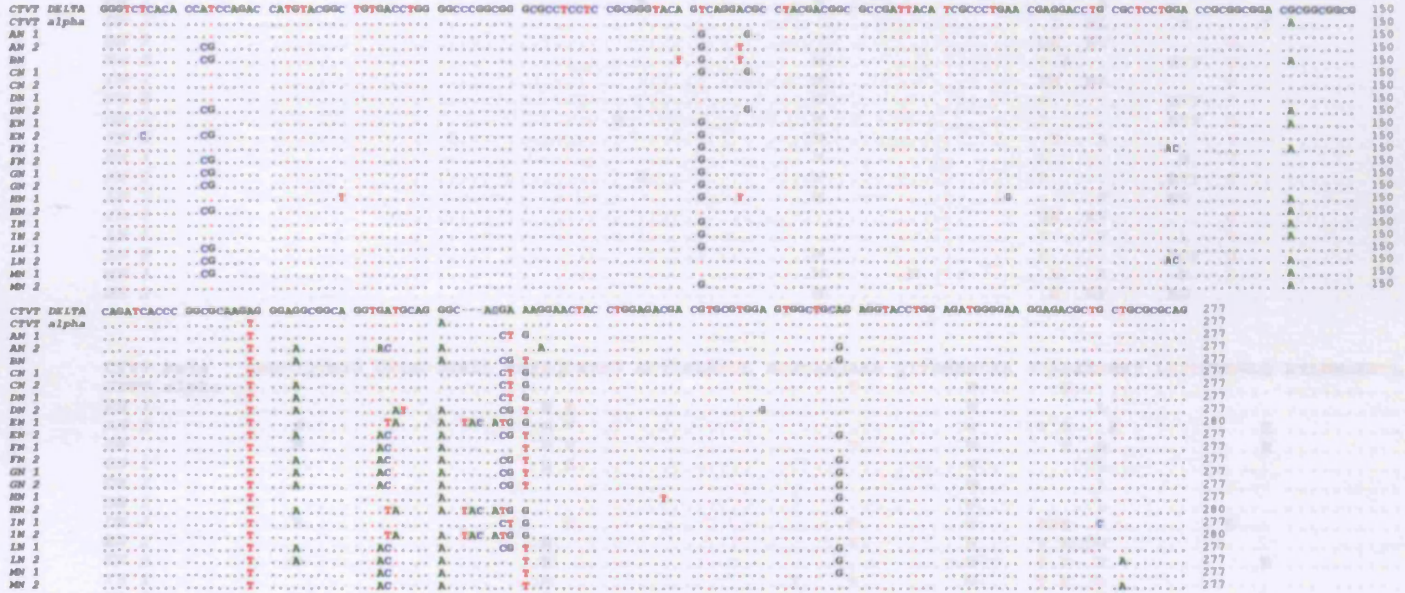


Fig 4.2b. DLA-88 exon 3 nucleotide alignment

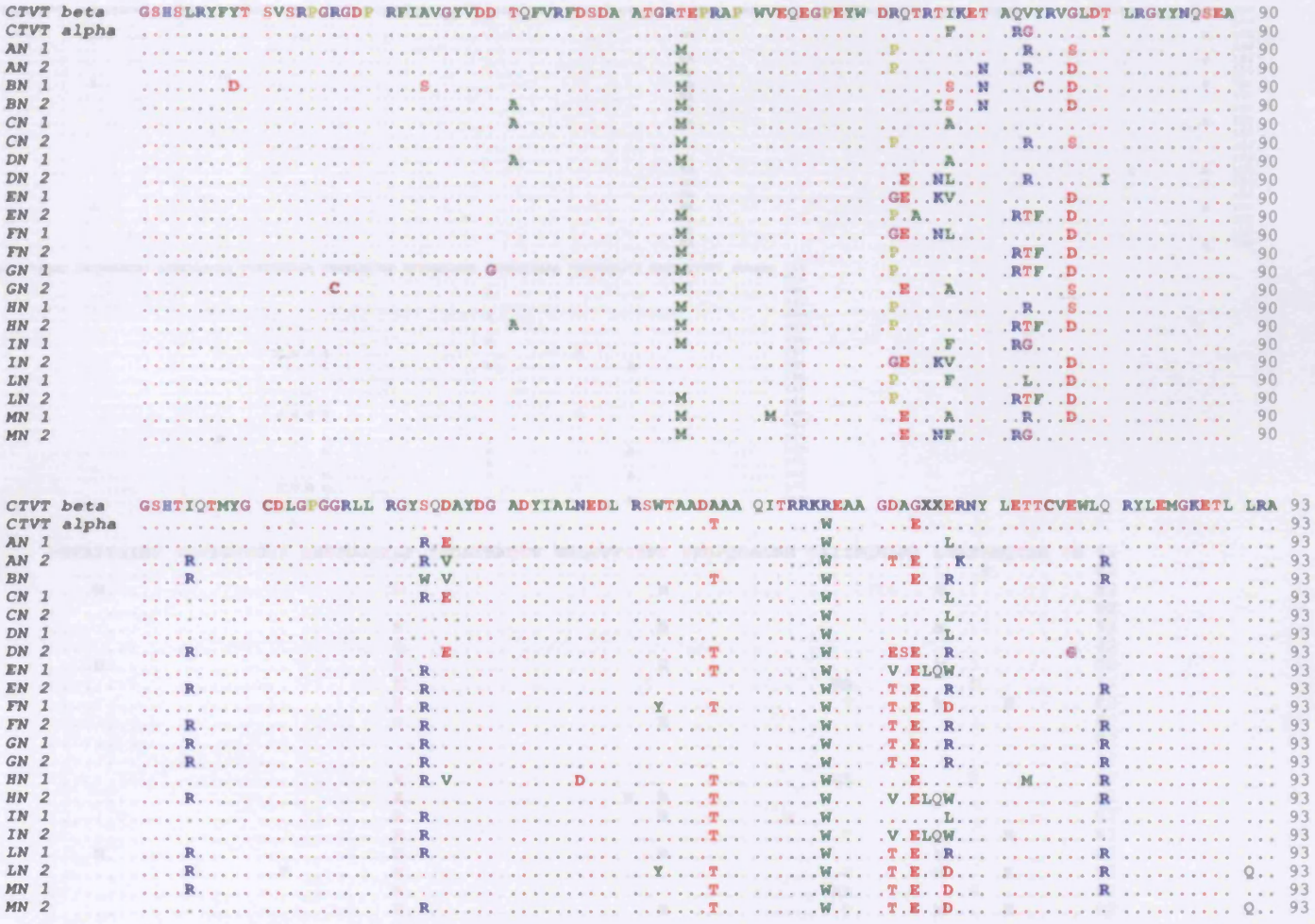
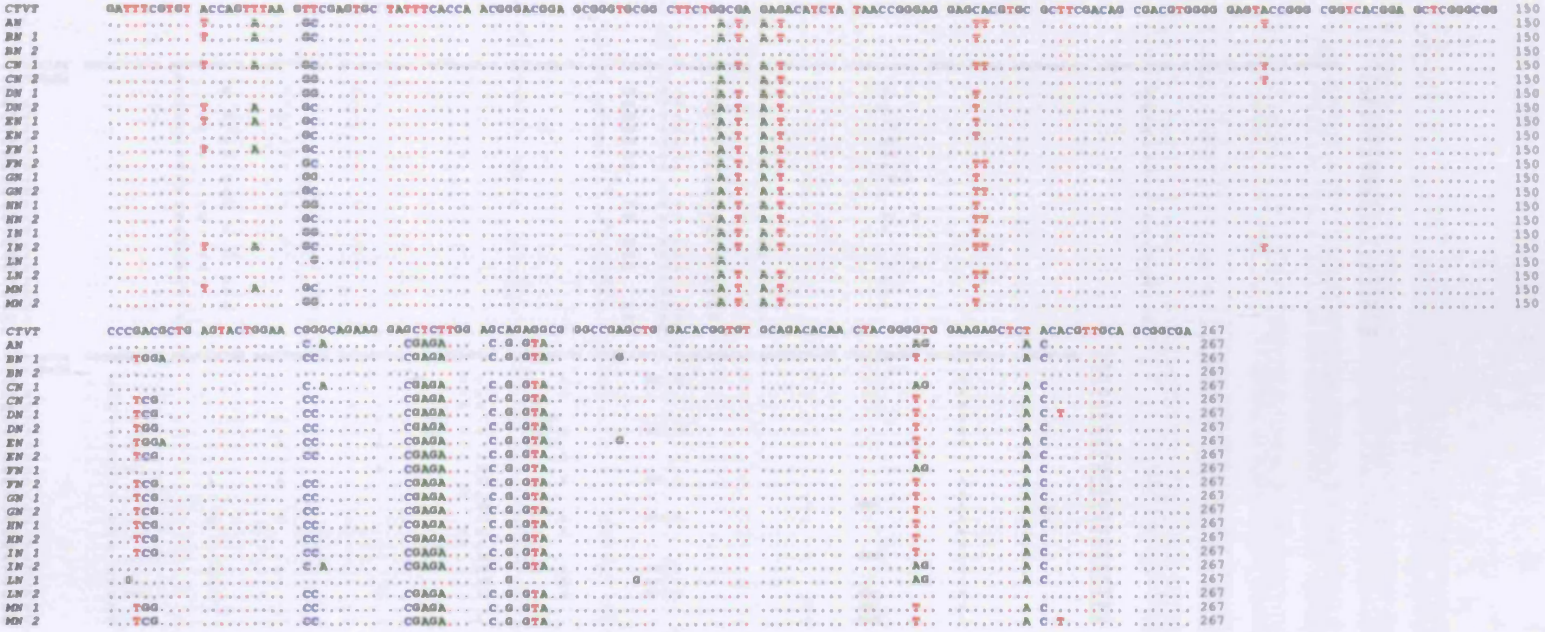
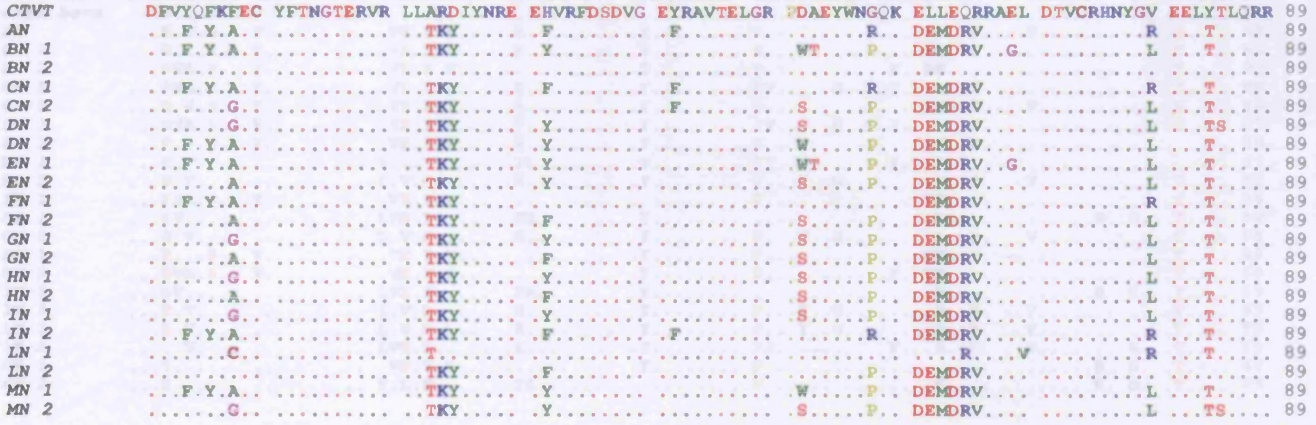


Fig 4.2c-d. DLA-88 exon 2 and exon 3 aminoacid alignment

Fig 4.2e-f. DLA-DQA1 exon 2 nucleotide and aminoacid alignment



g



h

Fig 4.2g-h. DLA-DQB1 exon 2 nucleotide and aminoacid alignment

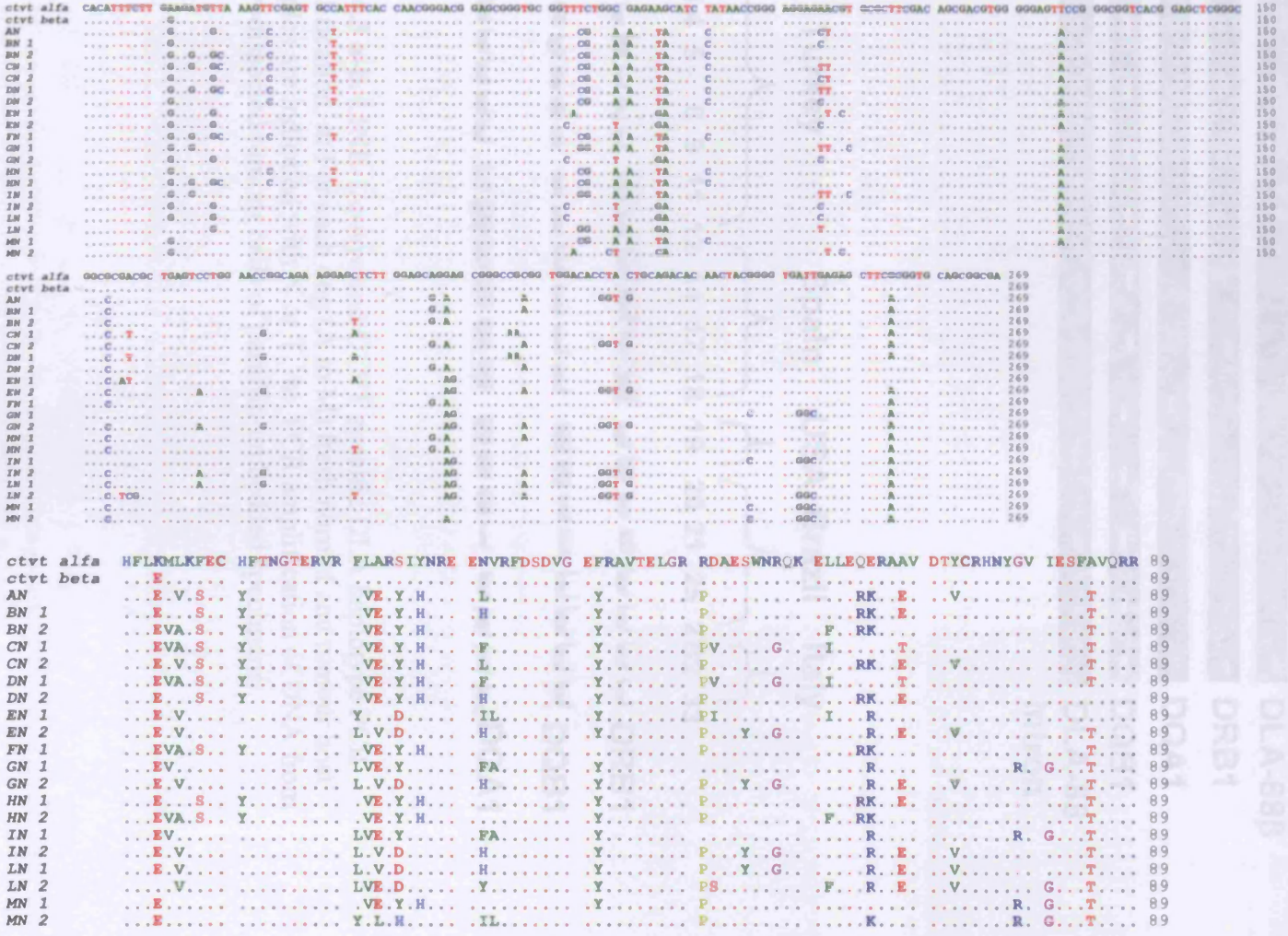


Fig 4.2i-j. DLA-DRB1 exon 2 nucleotide and aminoacid alignment

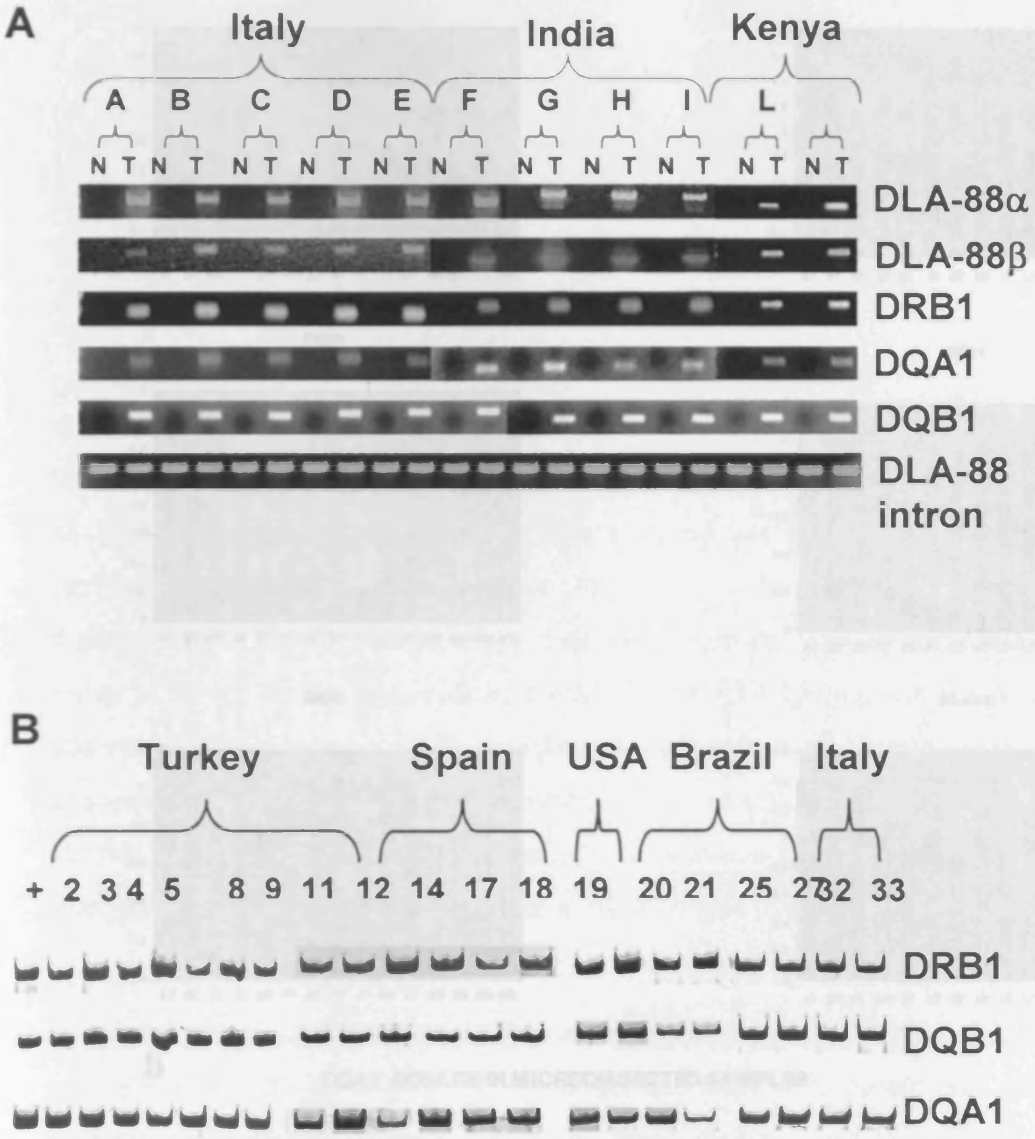


Fig 4.3 a-b. LINE-1/c-*myc* and CTVT specific DLA haplotype PCR amplification. **a**: For each dog (A to M) fresh normal and tumour host samples are indicated with N or T. **b**: PCR amplification of DNA from microdissected tumour cells of paraffin-embedded specimens.

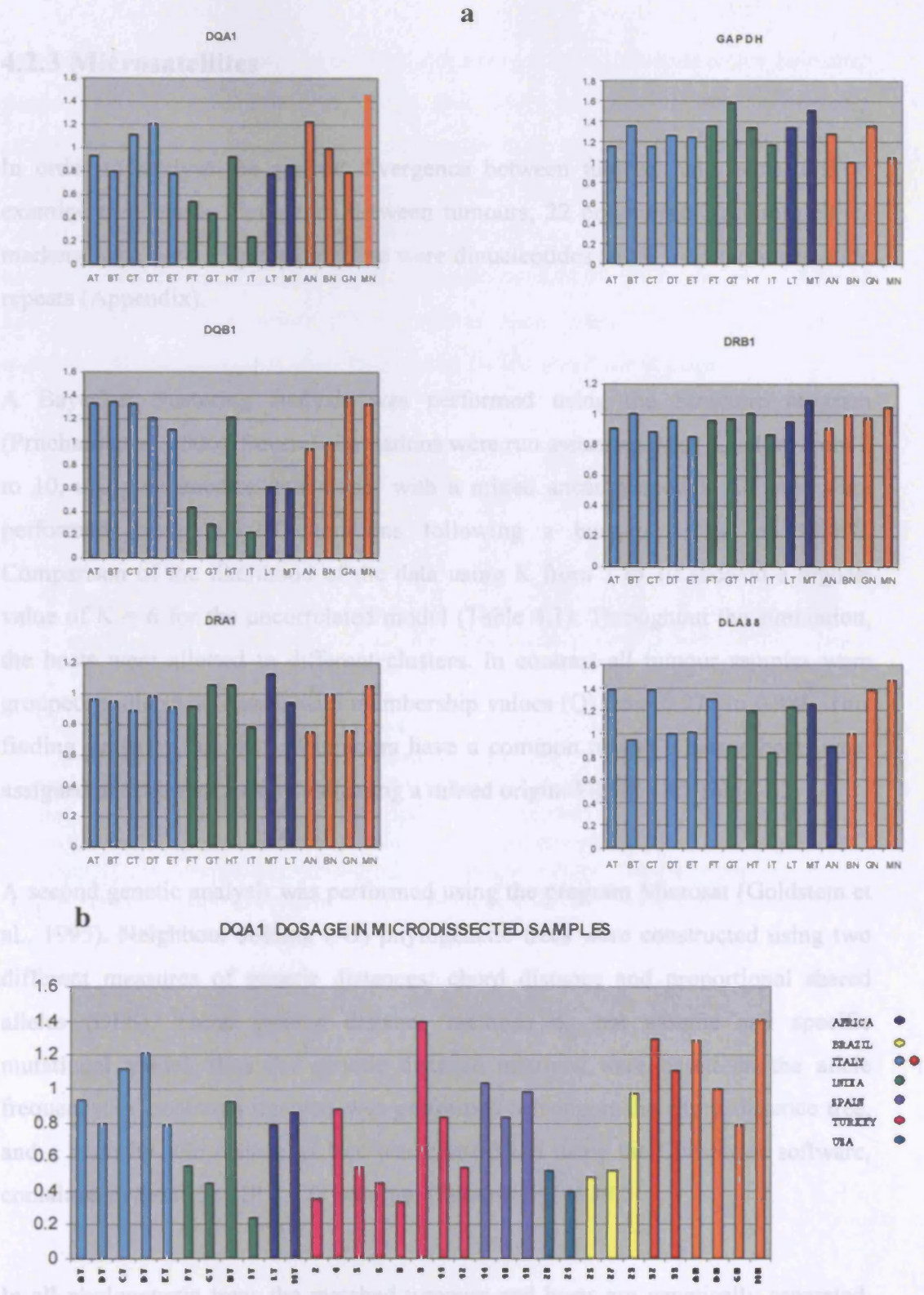


Fig 4.4. DLA gene dosage by SYBR-Green I Real-Time PCR. **a**, gene dosage detected in fresh tumour biopsies. **b**, DQA1 dosage comparison in fresh and fixed tumour biopsies. Orange bars represent the normal samples.

4.2.3 Microsatellites

In order to analyse the genetic divergence between tumours and hosts, and to examine the genetic divergence between tumours, 22 polymorphic microsatellites markers were used. Eighteen of these were dinucleotides and 4 were tetranucleotide repeats (Appendix).

A Bayesian clustering analysis was performed using the Structure program (Pritchard et al, 2000). Several simulations were run assuming prior K values from 2 to 10, using an uncorrelated model with a mixed ancestry model. All runs were performed using 100,000 iterations following a burn-in period of 10,000. Comparison of the likelihood of the data using K from 2 to 10 showed a highest value of K = 6 for the uncorrelated model (Table 4.1). Throughout the simulation, the hosts were allotted to different clusters. In contrast all tumour samples were grouped in the same cluster with membership values (Q) from 0.977 to 0.988. This finding suggests that that all tumours have a common origin, whereas hosts were assigned at different clusters indicating a mixed origin. Fig 4.5 and Table 4.2)

A second genetic analysis was performed using the program Microsat (Goldstein et al., 1995). Neighbour Joining (NJ) phylogenetic trees were constructed using two different measures of genetic distances: chord distance and proportional shared alleles (DPS). These genetic distance methods do not assume any specific mutational model, thus the genetic distance matrices were based on the allele frequency. A bootstrap analysis was performed to support the chord distance tree, and a majority-rule consensus tree was constructed using the Consensus software, contained in the PHYLIP 3.572 program (Felsenstein, 1989).

In all phylogenetic trees the matched tumours and hosts are genetically separated, with all tumours clustered together. This separation was supported in all 1000 bootstrap replicates. The chord and DPS trees show that the tumours have a recent

common ancestor compared to the host dogs (Fig 4.6 a-c). Despite a low bootstrap support, phylogenetic analysis showed that while all tumours were genetically related, they were also grouped according to their geographical origin, with the Italian and African tumours more closely related than the Indian tumours, with the exception of the Indian tumour sample H, which was closer to Italian and African tumours. This geographical separation was also evident for the hosts, where African and Italian dogs were more closely related than Indian dogs, again with the exception of the dog H that was closer to the Italian and African dogs.

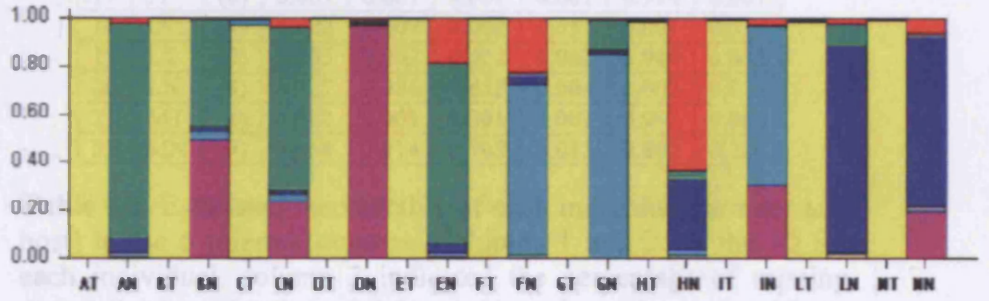


Fig 4.5. Structure analysis. Summary Plot of the estimated membership coefficients (Q) of individuals, in each cluster, assuming a model with admixture and uncorrelated frequencies, with K=6 (highest K value see table 4.1), without prior population information. The plotted result represent the run with highest posterior probability out of 5 independent runs with the same parameters. Each individual (N= normal; T= tumour) is represented by a single column, which is partitioned in to K colored segments representing the individual estimated membership fraction in each of the inferred K clusters.

K	Estimated Ln Prob of Data
2	-1250.3
3	-1197.9
4	-1173.8
5	-1168.0
6	-1143.8
7	-1178.8
8	-1169.8
9	-1208.8
10	-1253.6

Table 4.1. Estimates of posterior probabilities of the populations with K values from 2 to 10. Red arrow indicates the the highest posterior probability.

1	AT	(4)	0.001	0.001	0.001	0.001	0.994	0.001
2	AN	(0)	0.024	0.884	0.070	0.012	0.002	0.009
3	BT	(0)	0.002	0.001	0.001	0.001	0.993	0.001
4	BN	(0)	0.010	0.433	0.024	0.035	0.002	0.496
5	CT	(4)	0.002	0.002	0.002	0.016	0.968	0.010
6	CN	(0)	0.036	0.677	0.016	0.032	0.002	0.237
7	DT	(9)	0.001	0.002	0.001	0.001	0.993	0.001
8	DN	(0)	0.008	0.007	0.008	0.007	0.002	0.968
9	ET	(0)	0.001	0.001	0.001	0.001	0.994	0.001
10	EN	(4)	0.190	0.739	0.049	0.012	0.001	0.010
11	FT	(0)	0.001	0.001	0.001	0.001	0.993	0.001
12	FN	(9)	0.224	0.016	0.033	0.710	0.001	0.016
13	GT	(0)	0.001	0.001	0.001	0.001	0.994	0.001
14	GN	(0)	0.008	0.120	0.020	0.846	0.002	0.004
15	HT	(0)	0.002	0.003	0.002	0.002	0.989	0.002
16	HN	(0)	0.631	0.034	0.299	0.016	0.002	0.018
17	IT	(0)	0.001	0.001	0.001	0.001	0.994	0.001
18	IN	(4)	0.022	0.007	0.006	0.651	0.001	0.313
19	LT	(0)	0.005	0.002	0.002	0.001	0.989	0.002
20	LN	(4)	0.027	0.086	0.855	0.004	0.002	0.025
21	MT	(4)	0.002	0.001	0.001	0.001	0.993	0.001
22	MN	(9)	0.064	0.014	0.705	0.015	0.002	0.200

Table 4.2. Estimated membership of each individual (tumour and host) in the 6 inferred clusters. Columns 1 and 2 are the ID for each individual, column 3 indicated the percentage of missing data (allele), and columns 4-9 are the estimated membership in each inferred cluster or population

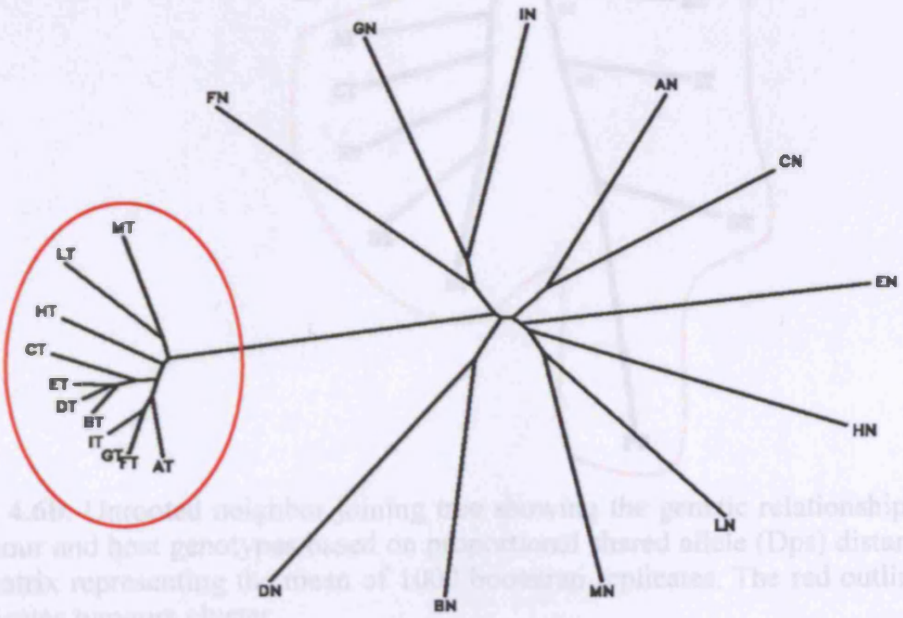


Fig 4.6a Unrooted neighbor joining trees showing the genetic relationship between tumour and host genotypes based on chord distance using a matrix representing the mean of 1000 bootstrap replicates.

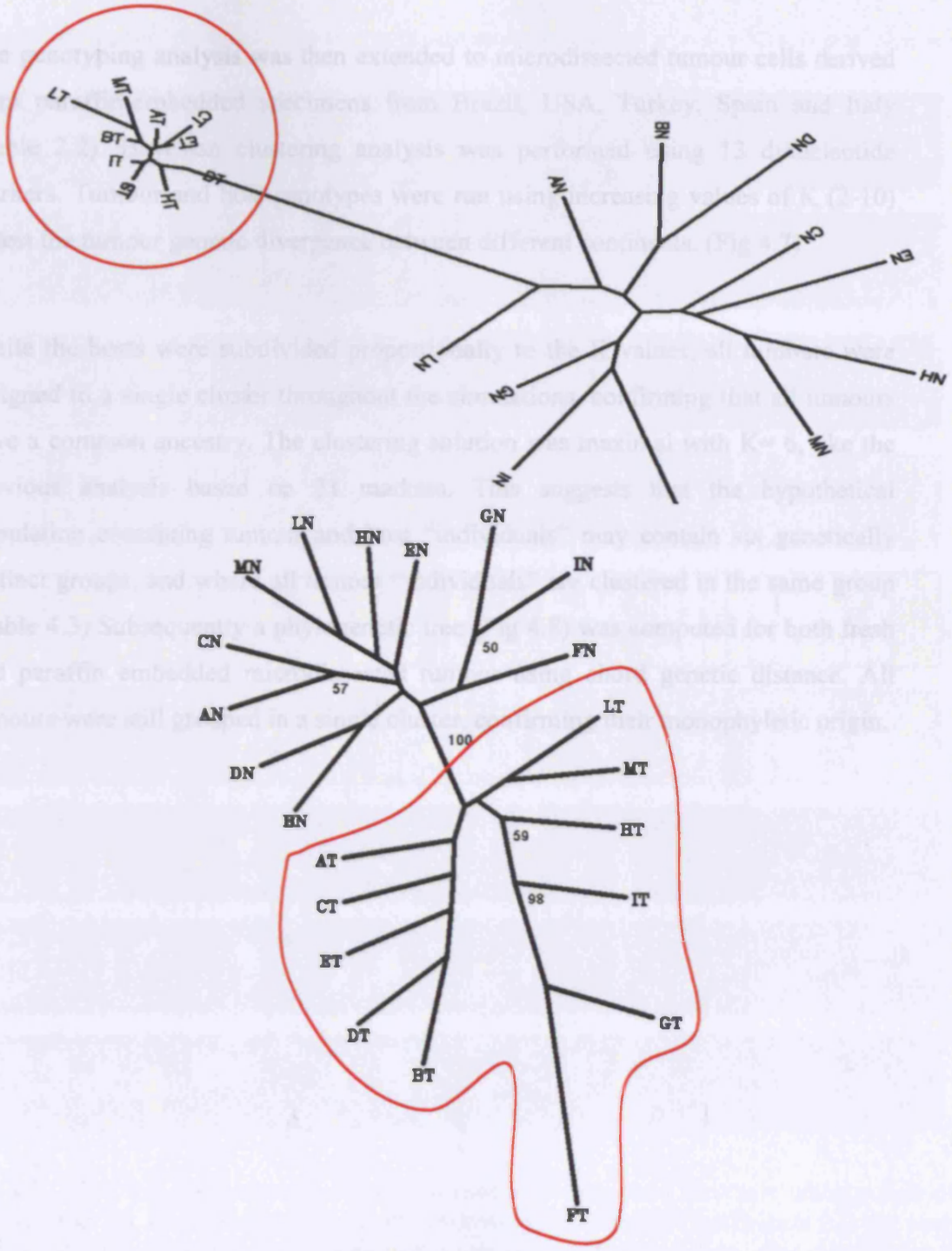


Fig 4.6b. Unrooted neighbor joining tree showing the genetic relationship between tumour and host genotypes based on proportional shared allele (Dps) distance, using a matrix representing the mean of 1000 bootstrap replicates. The red outlined circle indicates tumours cluster

Fig 4.6c. Unrooted Consensus NJ tree of tumours and hosts based on chord distance and 1000 bootstrap replicates. Bootstrap values are indicated at the corresponding node as percentage. Only bootstrap values over 50% are indicated. Branch lengths are proportional to bootstrap values. The red outline indicate tumour cluster.

The genotyping analysis was then extended to microdissected tumour cells derived from paraffin-embedded specimens from Brazil, USA, Turkey, Spain and Italy (Table 2.2). Bayesian clustering analysis was performed using 13 dinucleotide markers. Tumour and host genotypes were run using increasing values of K (2-10) to test the tumour genetic divergence between different continents. (Fig 4.7)

While the hosts were subdivided proportionally to the K values, all tumours were assigned to a single cluster throughout the simulations, confirming that all tumours have a common ancestry. The clustering solution was maximal with K= 6, like the previous analysis based on 21 markers. This suggests that the hypothetical population containing tumour and host "individuals" may contain six genetically distinct groups, and where all tumour "individuals" are clustered in the same group (Table 4.3) Subsequently a phylogenetic tree (Fig 4.8) was computed for both fresh and paraffin embedded microdissected tumour using chord genetic distance. All tumours were still grouped in a single cluster, confirming their monophyletic origin.

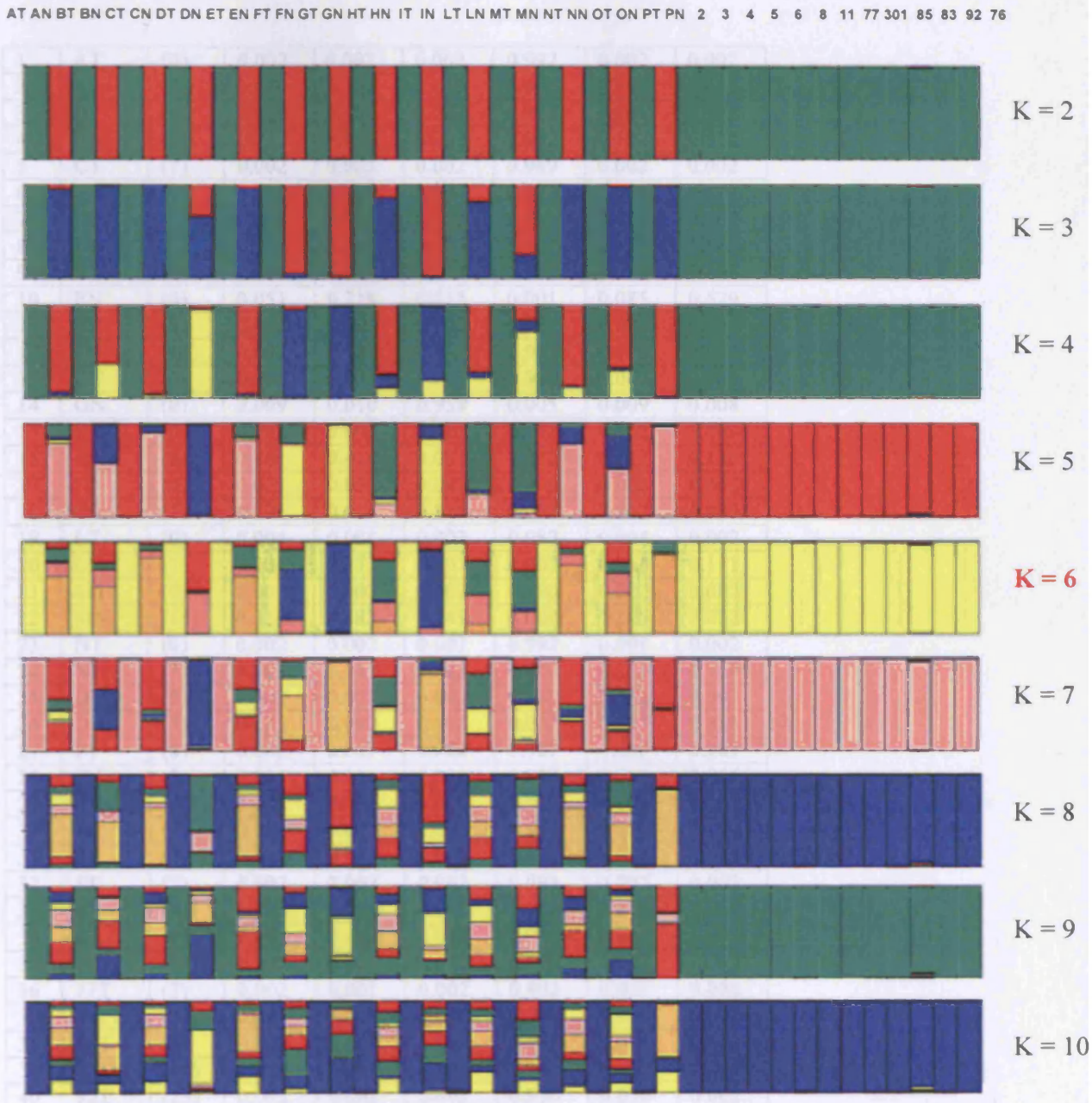


Fig 4.7 Clustering assignment based on ten independent runs of Structure using values of K from 2 to 10. Each Plot represents the estimated membership coefficients (Q) for each individual, in each cluster, assuming a model with admixture and uncorrelated frequencies, without prior population information. Each individual (N= normal; T= tumour) is represented by a single column, which is partitioned in to K colored segments representing the individual estimated membership fraction in each of the inferred K cluster. Bold red K value indicates the inferred K with highest posterior probability

1	AT	(0)	0.002	0.002	0.002	0.992	0.002	0.002
2	AN	(0)	0.076	0.125	0.019	0.002	0.146	0.631
3	BT	(0)	0.002	0.002	0.002	0.992	0.001	0.002
4	BN	(0)	0.233	0.059	0.003	0.002	0.191	0.512
5	CT	(7)	0.002	0.003	0.002	0.989	0.002	0.002
6	CN	(0)	0.034	0.056	0.007	0.002	0.074	0.827
7	DT	(15)	0.002	0.002	0.002	0.990	0.002	0.002
8	DN	(0)	0.532	0.013	0.006	0.002	0.432	0.015
9	ET	(0)	0.001	0.002	0.002	0.992	0.001	0.002
10	EN	(0)	0.051	0.218	0.015	0.001	0.085	0.629
11	FT	(0)	0.002	0.002	0.002	0.991	0.002	0.002
12	FN	(15)	0.092	0.187	0.561	0.002	0.137	0.021
13	GT	(0)	0.002	0.002	0.002	0.990	0.002	0.002
14	GN	(0)	0.009	0.010	0.959	0.005	0.009	0.008
15	HT	(0)	0.002	0.003	0.003	0.988	0.002	0.002
16	HN	(0)	0.201	0.418	0.031	0.002	0.212	0.136
17	IT	(0)	0.001	0.001	0.002	0.992	0.002	0.002
18	IN	(7)	0.091	0.016	0.819	0.002	0.067	0.005
19	LT	(0)	0.004	0.004	0.003	0.982	0.004	0.002
20	LN	(7)	0.208	0.357	0.007	0.002	0.316	0.111
21	MT	(0)	0.002	0.002	0.002	0.990	0.002	0.002
22	MN	(15)	0.319	0.382	0.049	0.002	0.225	0.022
23	NT	(0)	0.002	0.002	0.002	0.992	0.001	0.002
24	NN	(7)	0.082	0.043	0.003	0.002	0.121	0.749
25	OT	(0)	0.002	0.003	0.002	0.989	0.002	0.002
26	ON	(7)	0.235	0.092	0.008	0.002	0.227	0.436
27	PT	(0)	0.002	0.003	0.002	0.987	0.003	0.003
28	PN	(30)	0.005	0.114	0.008	0.002	0.005	0.866
29	2T	(0)	0.002	0.002	0.001	0.992	0.001	0.001
30	3T	(7)	0.002	0.002	0.002	0.991	0.002	0.002
31	4T	(7)	0.002	0.002	0.002	0.991	0.002	0.002
32	5T	(7)	0.002	0.002	0.002	0.991	0.002	0.002
33	6T	(0)	0.002	0.002	0.001	0.992	0.002	0.001
34	8T	(0)	0.003	0.003	0.002	0.988	0.003	0.002
35	11T	(7)	0.002	0.001	0.002	0.992	0.002	0.002
36	77T	(7)	0.002	0.001	0.002	0.992	0.001	0.002
37	301T	(15)	0.003	0.003	0.002	0.988	0.002	0.002
38	85T	(0)	0.006	0.004	0.002	0.982	0.003	0.002
39	83T	(15)	0.018	0.007	0.007	0.950	0.014	0.004
40	92T	(23)	0.002	0.002	0.002	0.990	0.002	0.002
41	76T	(0)	0.002	0.002	0.001	0.992	0.002	0.001

Table 4.3. Estimated membership of each individual (tumour and host) in the 6 inferred clusters with K= 6. Columns 1 and 2 are the ID for each individual, column 3 indicated the percentage of missing data (allele), and columns 4-9 are the estimated membership in each inferred cluster or population.

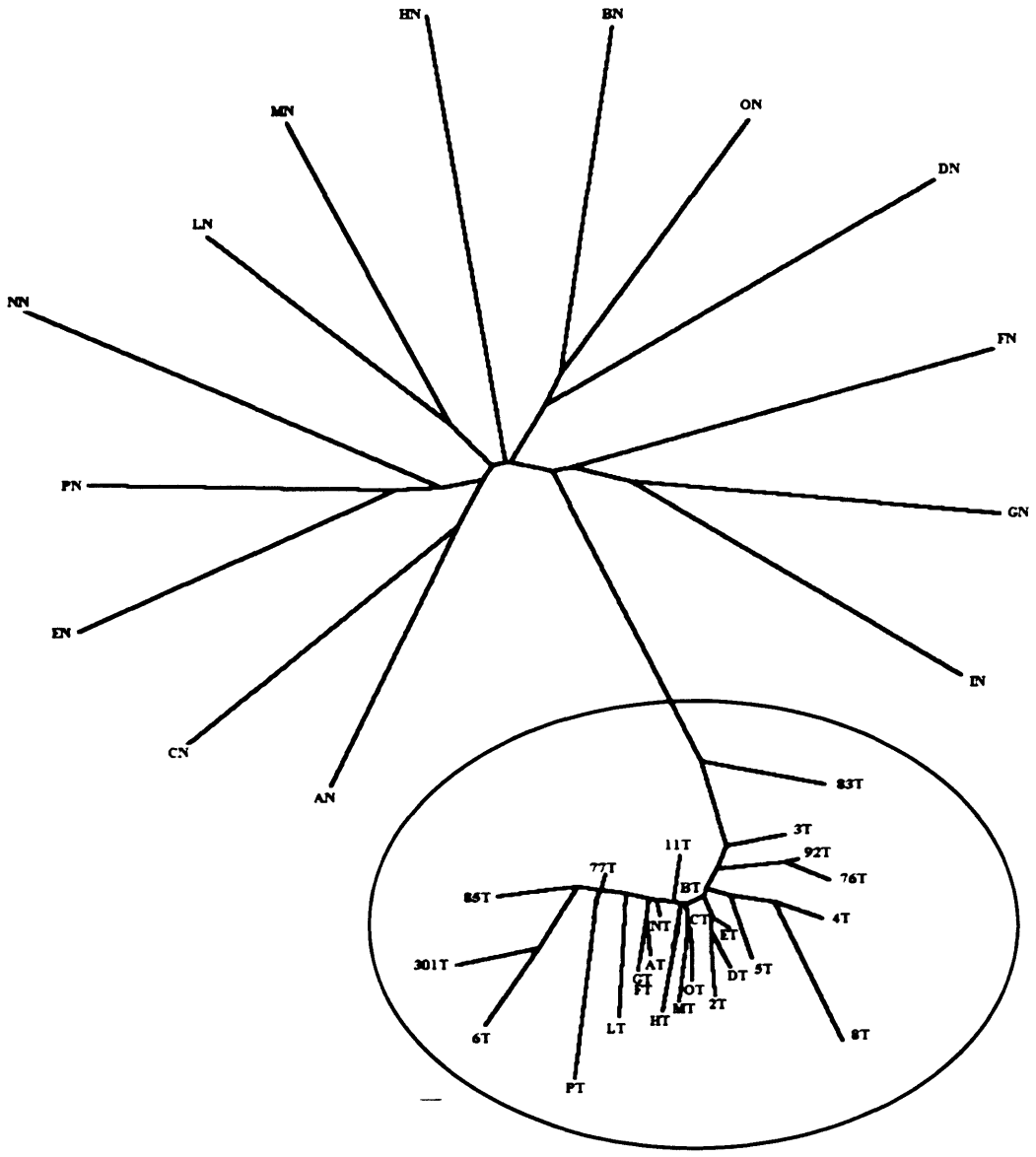


Fig 4.8. Unrooted neighbor joining tree showing the genetic relationship between tumours (in oval) and hosts genotypes based on a chord distance. The red outlined oval indicates tumours cluster.

4.2.4 Mitochondrial DNA

A 550 bp segment of the mt-DNA control region containing the hypervariable region 1 was amplified and sequenced in matched tumour and normal tissues (Fig 4.9). In addition a shorter 257 bp fragment within the hypervariable region were also sequenced in 21 microdissected tumour cells from paraffin embedded specimens (Fig 4.10). In all samples sequenced there were differences between the host haplotype and the tumour haplotype. Intra-tumour variation was detected, particularly in tumours G, H and L. To compare the genetic relationship between tumours and hosts and between different tumours, a minimum-spanning network was built using Network 4.0 software. All tumours were genetically distant from their hosts, and tumour divided in two clusters (Fig 4.11 a-b). Kenyan and Italian tumour haplotypes and also one Indian tumour sample (HT) were grouped in one cluster, while the remaining three Indian samples were grouped in the second cluster. This was the same Indian tumour that grouped with Kenyan and Italian tumours in microsatellite analysis.

To compare the tumour mt-DNA haplotypes with published haplotypes, we used a maximum likelihood method implemented in PAUP. As previously described, phylogenetic analysis of the mtDNA control region of wolves, dogs and coyotes identifies 5 phylogenetic groups or clades (Fig 4.12). Although the host haplotypes were distributed across clades A, B, and C, all tumour haplotypes were grouped in clade A. To date the majority of the wolf haplotypes are present in the clades B and C, and the only 3 wolf haplotypes present in clade A are clearly distant from the tumour haplotypes. This observation suggests that the tumour may have originated from a dog. However further analysis given in Chapter 7 indicates that a wolf origin of CTVT should not be precluded. Given the possibility that an ancestral tumour haplotype may be the origin of the 2 tumour mt-DNA haplotypes, I used the haplotypes present in all tumours, to reconstruct a hypothetical ancestral mt-DNA haplotype. The hypothetical ancestral tumour mt-DNA haplotype was included in the minimum spanning network representation (Fig 4.11b, 4.13a).

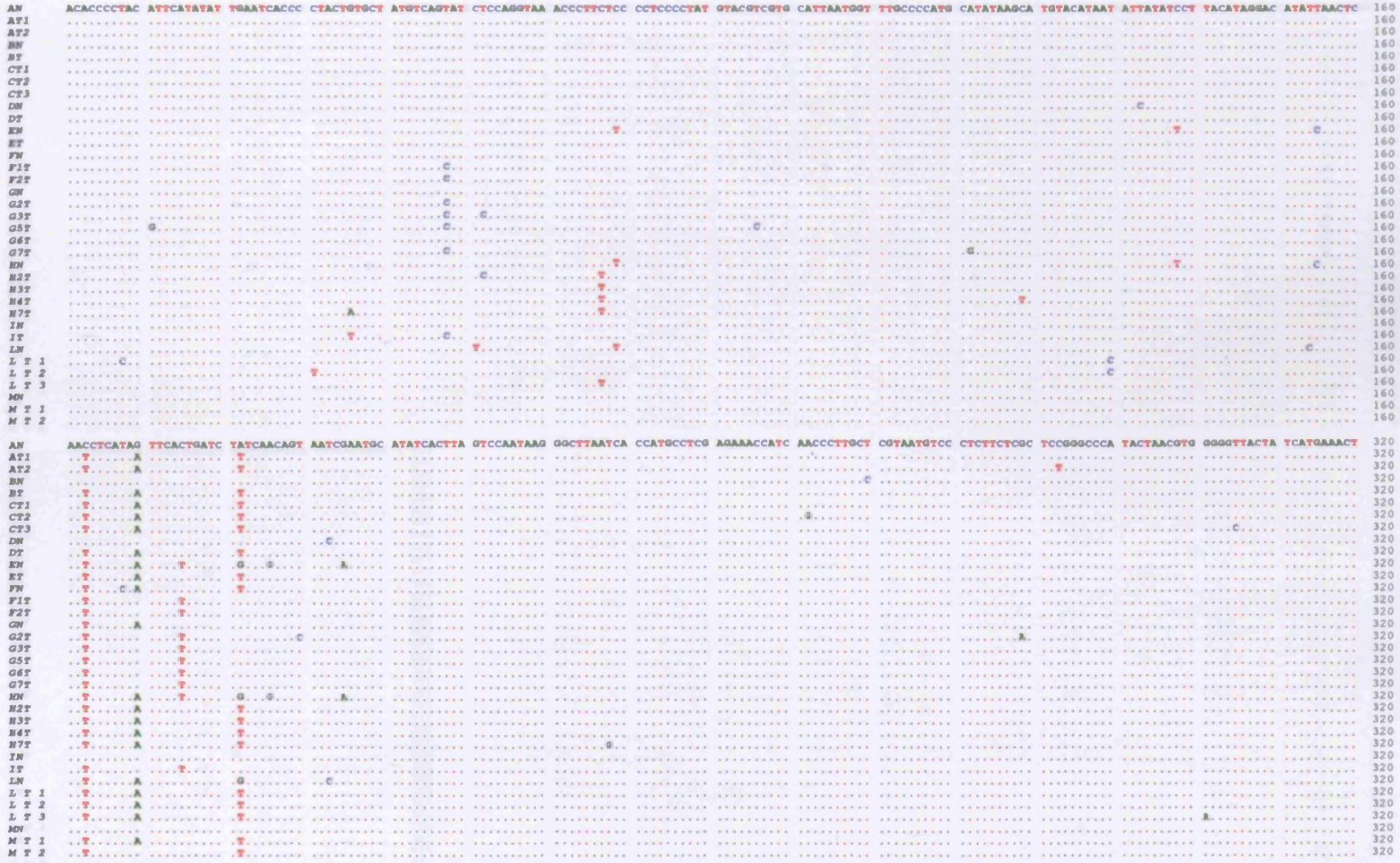


Fig 4.9. Mitochondrial DNA control region sequences alignment. Tumour and host haplotypes are indicated with T and N respectively. Tumour haplotype variant present in the same tumour are indicated with numbers

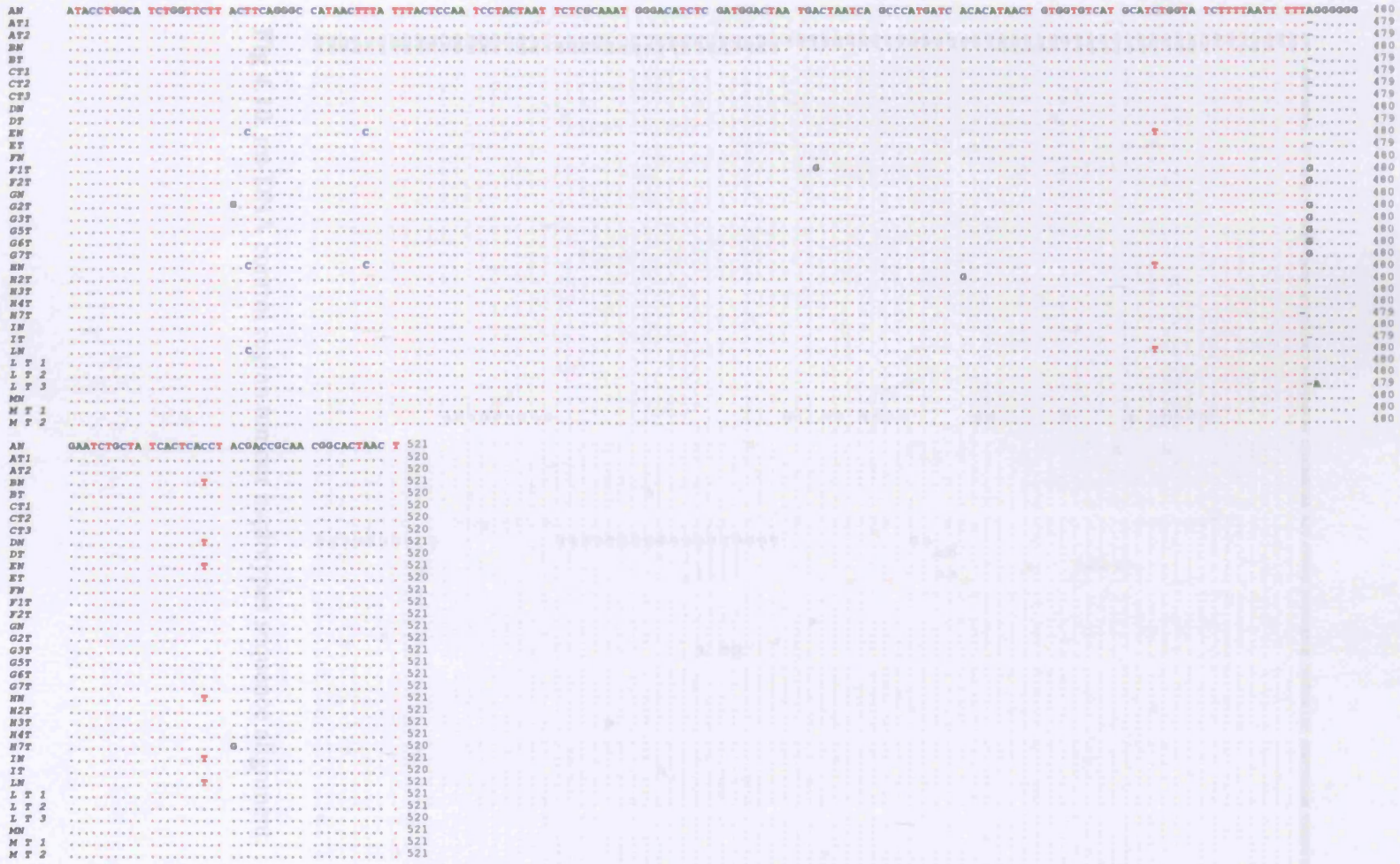


Fig 4.9. Mitochondrial DNA control region sequences alignment. Tumour and host haplotypes are indicated with T and N respectively. Tumour haplotype variants present in the same tumour are indicated with numbers.

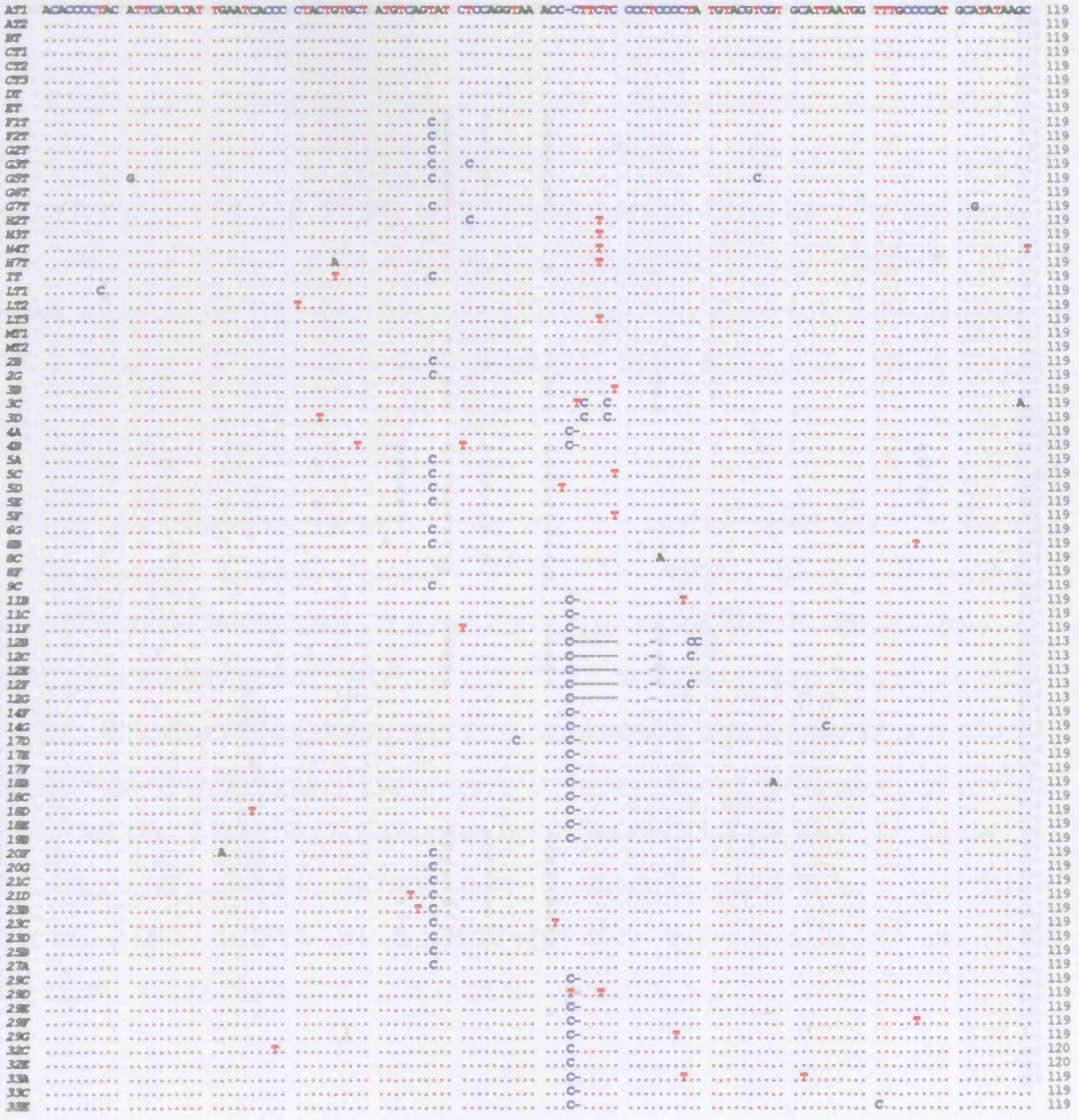


Fig 4.10. mt-DNA control region tumour haplotypes sequence alignment.

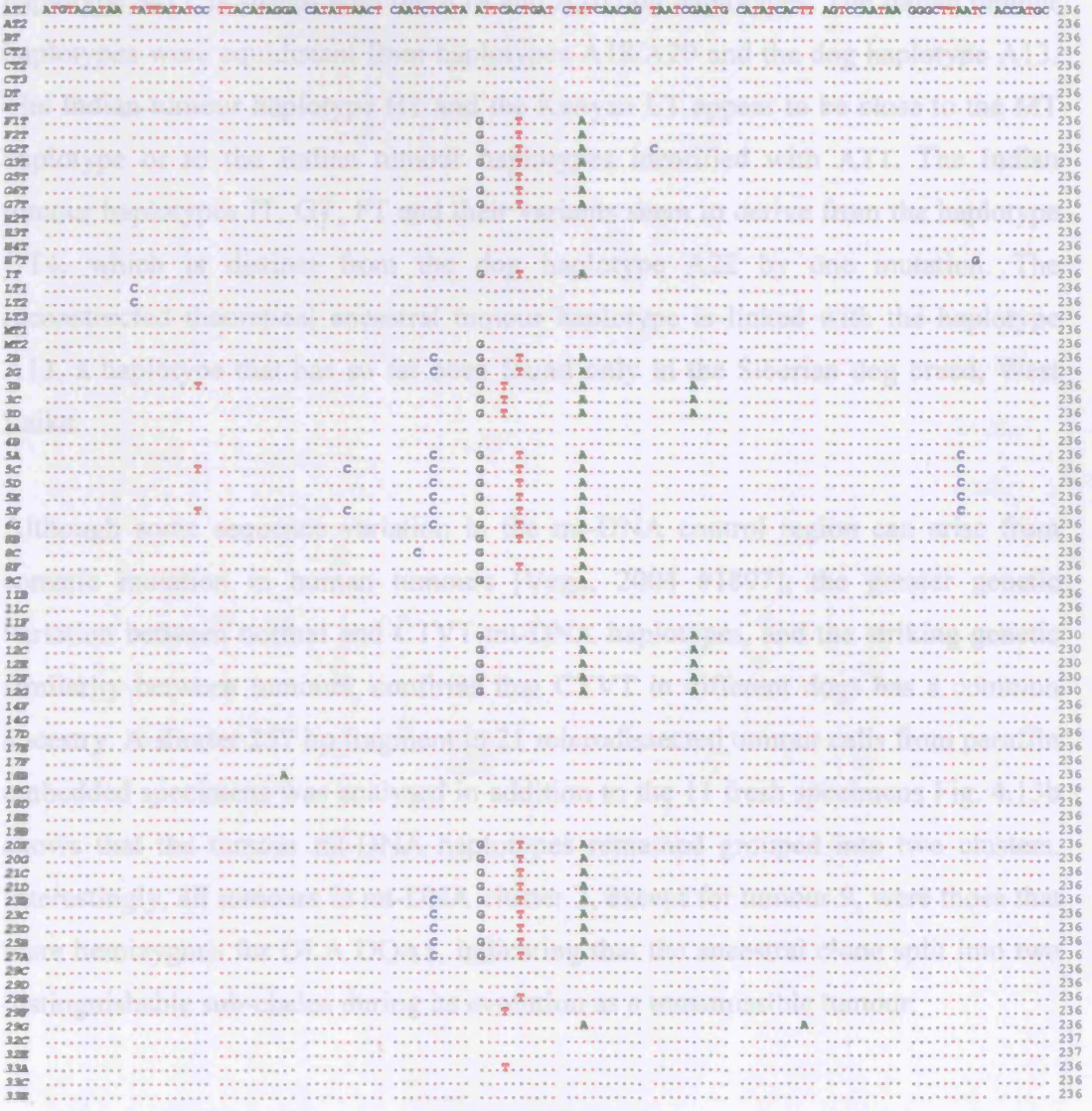


Fig 4.10. mt-DNA control region tumour haplotypes sequence alignment.

To resolve in more detail the genetic relationship between mt-DNA haplotypes present in clade A, a minimum spanning network was used (Fig 4.13a). The Kenyan haplotype M1T is shared with the A18 and A20 dog haplotypes. The Italian tumour haplotypes were equidistant from haplotypes A18/A20 and the dog haplotype A13. The Indian tumour haplotype HT and the Kenyan LT appear to be close to the MT haplotype or to the Italian tumour haplotypes identified with AT1. The Indian tumour haplotypes IT, GT, FT and their variants seem to derive from the haplotype GT4, which is distinct from the dog haplotype A22 by one mutation. The reconstructed theoretical ancestral tumour haplotype is linked with the haplotype A13, a haplotype that has so far been found only in the Siberian dog breed, West Laika.

Although some sequence variation in the mt-DNA control region can arise from somatic mutation in human tumours [Vega, 2004 #1897], the greater genetic variation between normal and CTVT mt-DNA haplotypes, and the striking genetic similarity between tumours, confirms that CTVT in different dogs has a common ancestry. A shorter 257 bp fragment in 21 microdissected tumour cells from paraffin embedded specimens was analysed in addition to the 11 fresh specimens Fig. 4.13b shows that the tumour mt-DNA haplotypes remained grouped into two clusters. Interestingly, all tumours in mt-DNA cluster 2, except for tumour 9, were those that were hemizygous for DLA DQA1, indicating that the ancestral clone split into two distinguishable sub-clades during its evolution as a transmissible tumour.

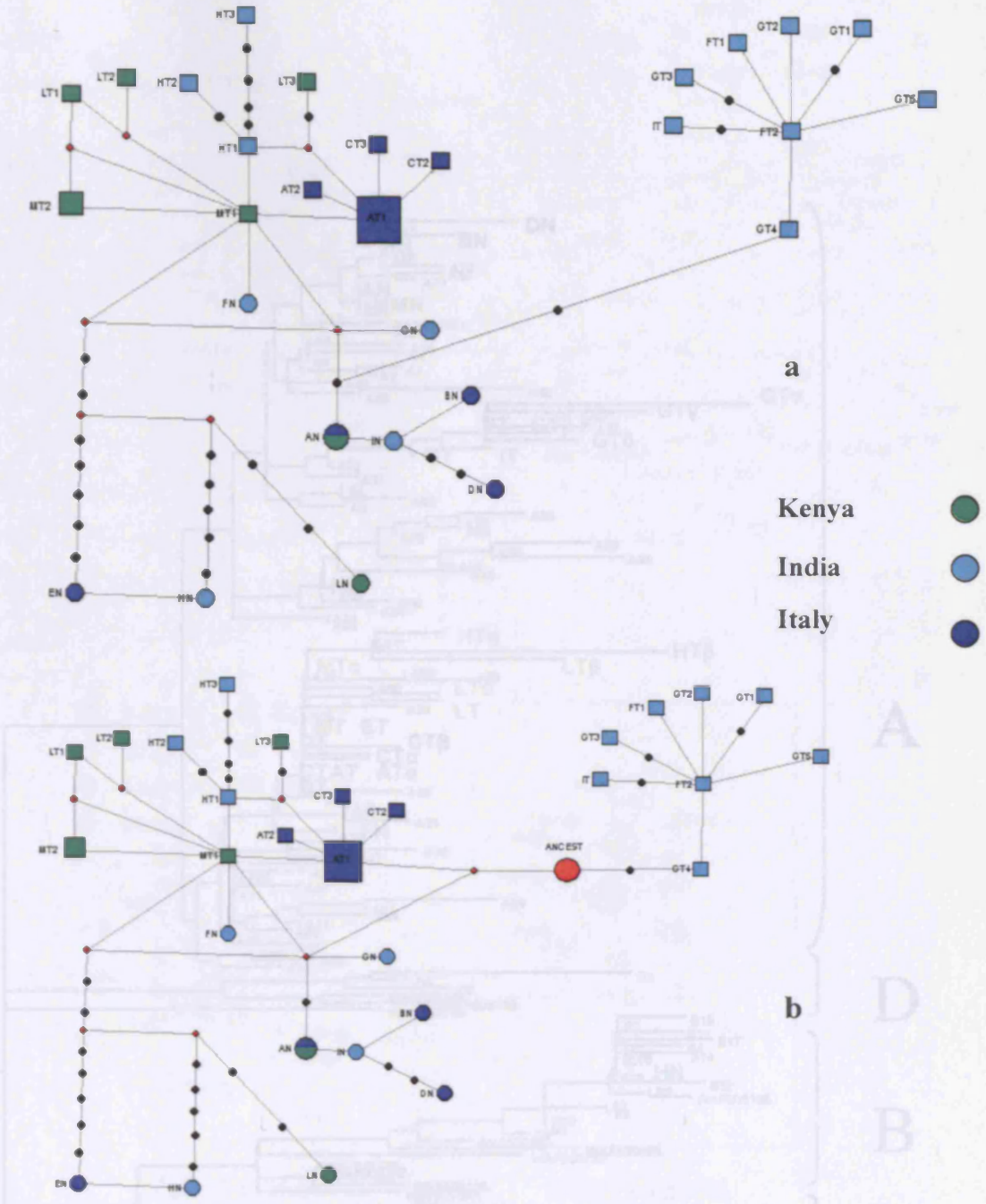


Fig 4.11. Minimum-spanning networks representing the genetic relationship between mt-DNA haplotypes found in tumors and their hosts. **Fig a-b** represent networks without and with inferred tumour ancestral haplotype respectively. Each branch represents a 1 bp change or indel, dots represent hypothetical intermediates. Tumor haplotypes are indicated in squares, circles represent the host haplotypes, red circle represents the tumor ancestral haplotype. The red lines represent the link in the presence of the tumor ancestral haplotype. Haplotypes found in India, Kenya, and Italy are indicated with green, blue, pale blue respectively. Tumor haplotype AT1 contains the haplotypes found in tumors B, C, D, and E. Tumor haplotype variants present in the same tumor are indicated with numbers

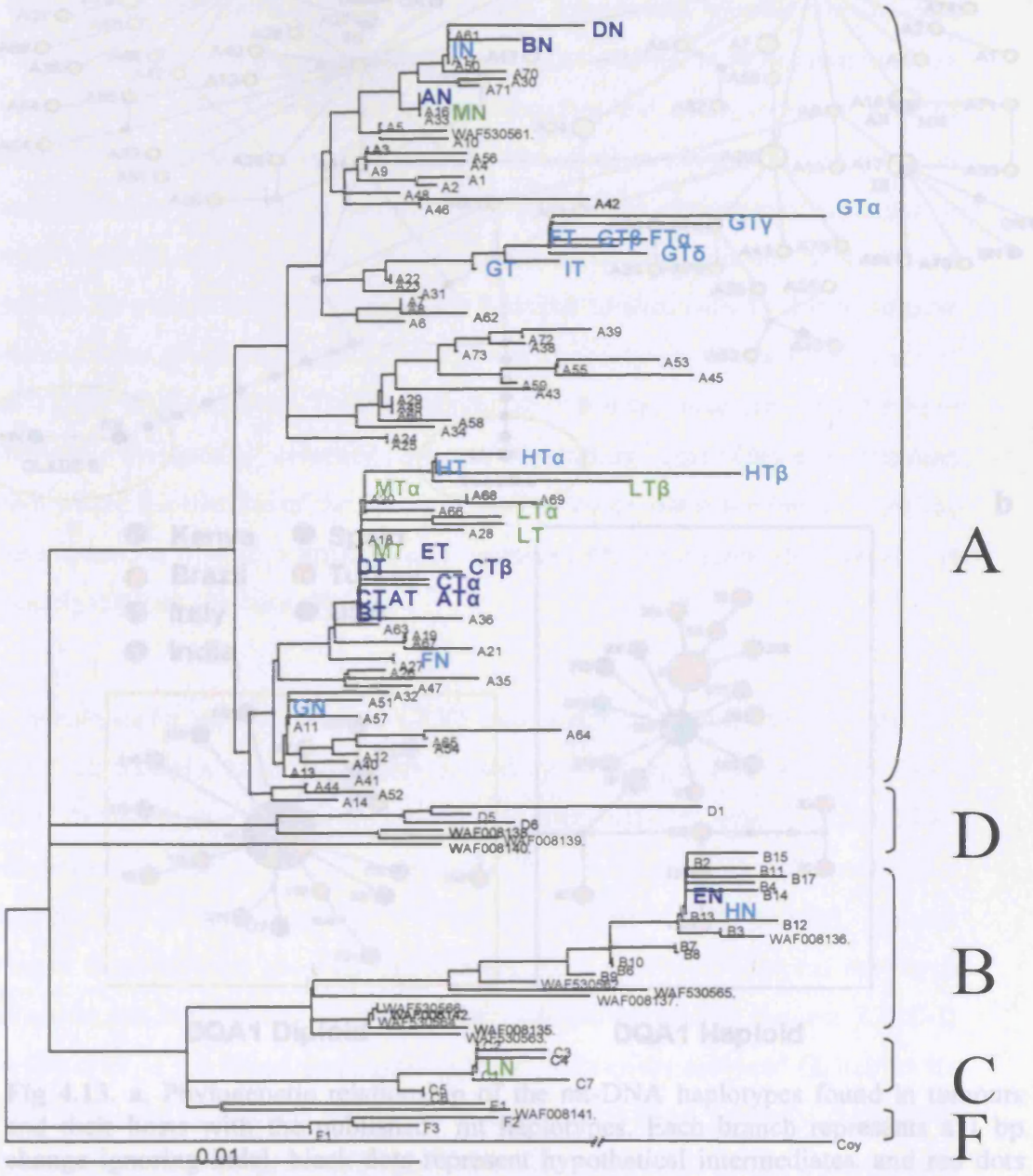


Fig 4.12. ML Phylogenetic tree of mt-DNA control region. Tumour and host sequences are indicated with T and N respectively with different colours indicating their origin: blue (Italy), blue pale (India), green (Kenya). A, B, C, D F, represent the 5 inferred clades

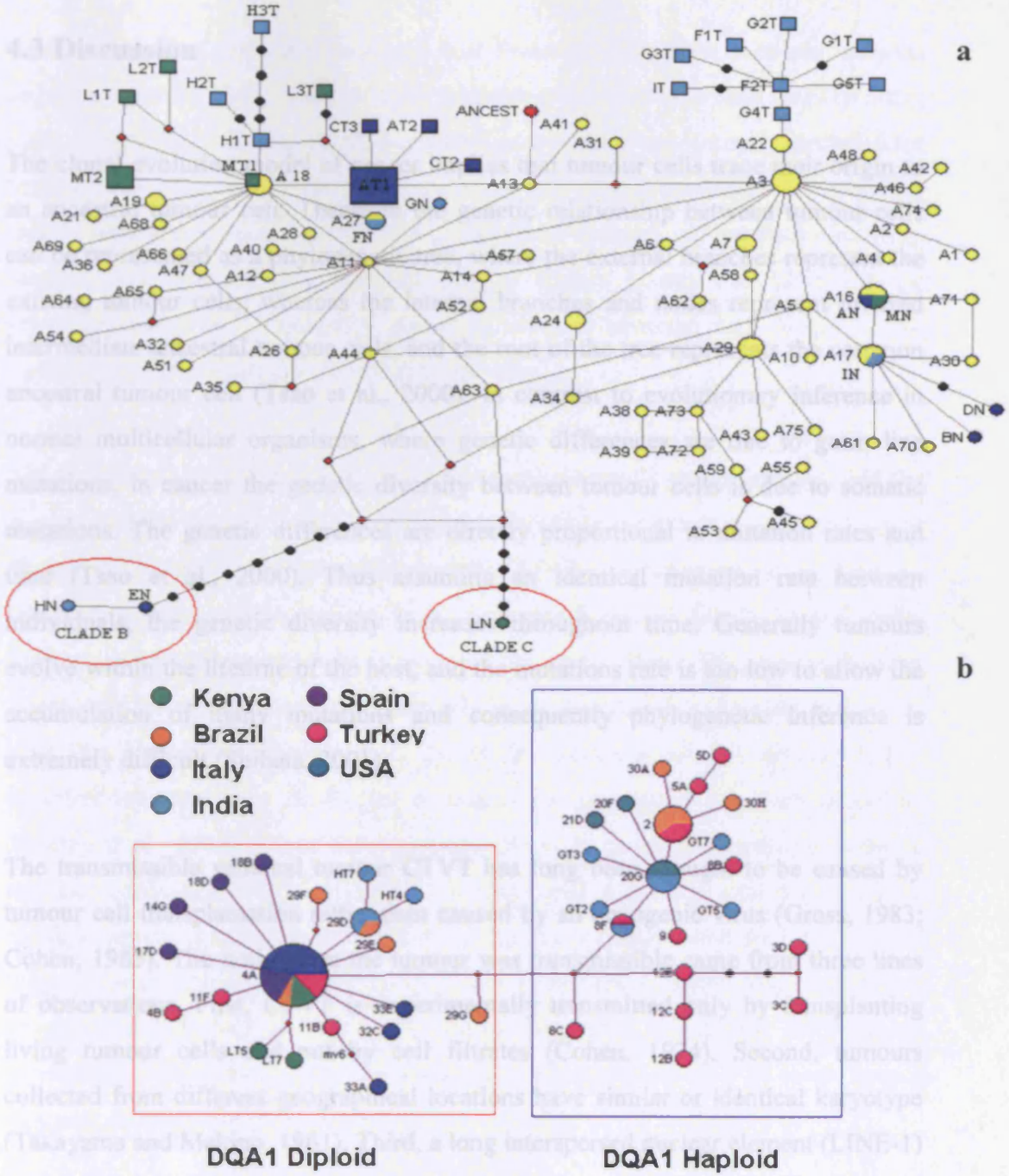


Fig 4.13. a, Phylogenetic relationship of the mt-DNA haplotypes found in tumours and their hosts with the published mt haplotypes. Each branch represents a 1 bp change ignoring indel, black dots represent hypothetical intermediates, and red dots represent median vectors. The squares and circles represent the tumour and host haplotype, yellow circles indicate the published haplotypes. The red circle represents the inferred hypothetical tumour ancestral sequence. Squares and circles are proportional to the number of tumours sharing the same haplotype. **b,** Phylogenetic network based on a 236 bp sequences of mtDNA haplotypes found in tumours from fresh and microdissected tumour samples. The red and blue outlined squares indicate tumours samples with homozygous and hemizygous DQA1 pattern.

4.3 Discussion

The clonal evolution model of cancer implies that tumour cells trace their origin to an ancestral tumour cell. Therefore the genetic relationship between tumour cells can be represented as a phylogenetic tree, where the external branches represent the existing tumour cells, whereas the internal branches and nodes represent inferred intermediate ancestral tumour cells, and the root of the tree represents the common ancestral tumour cell (Tsao et al., 2000). In contrast to evolutionary inference in normal multicellular organisms, where genetic differences are due to germ line mutations, in cancer the genetic diversity between tumour cells is due to somatic mutations. The genetic differences are directly proportional to mutation rates and time (Tsao et al., 2000). Thus assuming an identical mutation rate between individuals, the genetic diversity increases throughout time. Generally tumours evolve within the lifetime of the host, and the mutations rate is too low to allow the accumulation of many mutations and consequently phylogenetic inference is extremely difficult (Shibata, 2001).

The transmissible venereal tumour CTVT has long been thought to be caused by tumour cell transplantation rather than caused by an oncogenic virus (Gross, 1983; Cohen, 1985). The notion that the tumour was transmissible came from three lines of observations. First, CTVT is experimentally transmitted only by transplanting living tumour cells and not by cell filtrates (Cohen, 1974). Second, tumours collected from different geographical locations have similar or identical karyotype (Takayama and Makino, 1961). Third, a long interspersed nuclear element (LINE-1) insertion near to the c-*myc* gene was found in all tumours analyzed (Katzir et al., 1987). However a detailed molecular genetic analysis of naturally transmitted tumours has not been previously performed to resolve the genetic relationship between tumour and its host and neither has the genetic diversity been examined between tumours collected worldwide. In order to analyse the genetic relationship between tumours and host and to determine the genetic divergence between

tumours, matched tumour tissues and host blood samples from naturally affected dogs were analysed for the first time, collected from 16 unrelated dogs in Italy, India, Kenya. In addition, 21 tumour paraffin embedded specimens collected from Brazil, USA, Turkey, Spain and Italy were analysed. Consistent with previous reports obtained from experimentally transplanted tumours, all naturally occurring tumours but none of the matched normal tissues posses the LINE-1/c-*myc* rearrangement. The 21 naturally occurring tumours obtained from Brazil, USA, Turkey, Italy and Spain also shared this insertion.

Although this LINE–1 insertion might represent a somatic rearrangement, and the presence of this marker in all tumours would therefore indicate a monoclonal origin (Katzir et al., 1987), the possibility that this LINE-1 insertion might represent an inherited genetic marker present in a particular breed or family is still open. Recently a LINE-1 insertion in intron 5 of the canine factor IX gene, in German Wirehaired Pointers affected by haemophilia B, was found to be inherited for at least five generation (Brooks et al., 2003). In humans a non-deleterious LINE-1 insertion inherited in a family for at least three generation has been described (Conley et al., 2005). Given these considerations, to provide compelling evidence for the monoclonal origin of CTVT, I considered a genetic analysis based on multiple genetic markers to be essential. In this study I have tested the CTVT clonal origin by analysing different polymorphic DNA markers including four DLA genes, 21 microsatellite loci and mt-DNA control regions. I found that tumours are genetically distinct from their hosts and that all worldwide CTVTs arose from a common ancestral neoplastic clone.

According to the clonal evolution theory of cancer, tumour cells evolve through selection and clonal expansion (Nowell, 1976; Greaves, 2002). Experimental and theoretical studies have suggested that in order to evolve, tumour cells must acquire an increased mutation rate (Loeb and Loeb, 2000). Several studies have shown that tumours with defects in the mismatch repair system have an elevated mutation rate.

As a consequence of genetic instability, tumour cells are expected to diverge during tumour progression. Thus to analyse the clonal evolution of tumours it is necessary to use several genetic markers with different mutation rates (Garcia et al., 2000). In phylogenetic analysis genetic markers with low mutation rates are used to infer ancestral divergence and markers with high mutation rates are used to infer recent divergence (Ridley, 2004a).

In this study I found that CTVT samples from 5 continents share the same sequences in four polymorphic DLA genes, but that using more highly polymorphic markers such as mt-DNA and microsatellites, tumours showed a partial clonal divergence. During its evolution the original clone has diverged in two subclades according to mt-DNA clusters and loss of alleles in DLA DQA1.

Although tumour mt-DNA divergence might suggest two separate origins, the DLA and c-*myc* results support a monoclonal origin. This divergence may be also explained, if one assumes that in the original tumour more mt-DNA haplotypes were present (heteroplasmy). Heteroplasmy of mt-DNA has been described in physiological conditions related to aging and in cancer (Michikawa et al., 1999; Bianchi et al., 2001). It is also a well known phenomenon in neurological diseases caused by mt-DNA deletion/mutations (Fassati et al., 1994). It is also conceivable that this tumour on rare occasions might acquire host mt-DNA; the tumour has phagocytic phenotype and could take up cytoplasmic fragments from neighbouring host cells. However, it would be an even rarer event for mt-DNA introduced in this way to become the dominant population or achieve homoplasmy. The lack of intermediate tumour haplotypes between the 2 tumour clusters may be due to mitochondrial genetic drift during cell division and also to a bottleneck effect during transplantation between dogs, because only a small number of tumour cells will be implanted in the next host. Given the impossibility to obtain the normal counterpart of the paraffin embedded tumour samples, the mt-DNA heteroplasmy in these samples might be also due to the presence of normal mt-DNA haplotypes.

The fact that tumours across five continents maintain the sequence of 4 polymorphic MHC genes unmutated seems to suggest that the MHC genes are under selection. One the most peculiar features of CTVT is its natural transplantability. This ability has been suggested to be due to the fact that the tumour cells do not express the DLA class I and II antigens during the progressive stage of tumour growth (Yang et al., 1987). According to somatic evolution, tumour cells evolve towards the selfishness that ultimately results in a more malignant phenotype with a severe effect on the host's fitness (Greaves, 2002; Michor et al., 2004). During progression tumour cells frequently evade immunosurveillance by down-regulating the MHC antigen expression (Dunn et al., 2004a). In contrast to parasite evolution, the host conditions are extremely important, and virulence has to be modulated to limit damage to the host particularly if host fitness is necessary to maximize the transmission to a new host (Frank, 1996). Although CTVT does not express MHC class I and II antigens during the progression stage, it does express MHC antigens during the regression stage (Yang et al., 1987), thus acting as a relatively benign parasite in the immune competent host.

Recently it has been shown that mt-DNA mutation rates in tumours reflect the neutral evolution of mt-DNA in the normal human population (Vega et al., 2004). In this study I found that the mutations present in the mitochondrial control region of the tumours are common to mutational hotspots described in normal dog populations. These results indicate that the mt-DNA divergence between the 2 subclades might be due to genetic drift rather than selection.

Generally tumours evolve within the lifetime of the host, and the mutation rate is too low to allow the accumulation of many mutations and consequently phylogenetic inference is difficult. Due to their high mutation rates, microsatellites markers are extremely useful in phylogenetic studies. Several studies have shown

that tumours with defects in the mismatch repair system have an elevated mutation rate, especially in microsatellites (Atkin, 2001).

It generally thought that tumour genetic instability increases throughout progression, so that tumour cells evolve toward a genetically instable phenotype (Loeb and Loeb, 2000; Nowak et al., 2005). Genotyping results based on 21 microsatellite loci indicate that CTVT is genetically stable and suggest that the moderate genetic diversity present between tumours from different geographical locations is the result of normal mutation rates. Although CTVT is highly aneuploid, the karyotype is remarkably constant among tumours from USA, Africa and Japan (Cohen, 1985). Therefore its genomic rearrangement appears to have stabilised early in its emergence as a transmissible parasite. My results indicate that CTVT during its evolution has stabilised its genome in order to maintain the parasite features. It is generally accepted that cancer evolves to increase its independence from the host and to became more selfish (Nunney, 1999). However CTVT cells with their stabilised genome may reflect kinship selection and reduced virulence, thus aiding host survival and onward tumour transmission. The decreased pathogenicity may be also favoured by the fact that during transmission only a small proportion of tumour cells implant in the new host, thus decreasing the genetic variability within the tumours and therefore the competition between tumour cells having different fitness advantages. The results obtained in this study seem to contrast to the common view that cancer evolution progressively leads to an increasingly genetically instable and selfish clone.

Samples	Origin	Name	1	1	2	2	8	8	9	9	28	28	31	31	38	38	42	42	53	53	66	66	68	68	92	92
TUMOUR	ITALY	AT	158	158	171	171	169	169	164	174	134	134	132	132	152	158	123	123	178	178	179	179	140	140	145	145
HOST	ITALY	AN	158	158	177	185	165	174	168	170	130	130	134	134	168	165	143	140	159	161	179	179	141	166	158	160
TUMOUR	ITALY	BT	158	158	171	171	163	163	164	174	134	134	132	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	ITALY	BN	158	158	183	185	180	172	168	172	128	130	129	136	160	163	146	148	159	159	181	179	137	159	158	158
TUMOUR	ITALY	CT	158	158	171	171	163	169	164	174	134	134	132	134	152	158	123	123	142	178	0	0	140	140	145	145
HOST	ITALY	CN	158	158	183	160	174	172	166	172	130	136	134	138	165	165	140	142	171	189	179	179	160	160	158	160
TUMOUR	ITALY	DT	158	158	171	171	163	163	0	0	134	134	134	134	0	0	123	123	178	178	179	179	140	140	145	145
HOST	ITALY	DN	158	158	187	185	182	182	162	164	128	138	129	129	168	160	146	146	189	171	181	184	159	178	158	166
TUMOUR	ITALY	ET	158	158	171	171	163	163	164	174	134	134	132	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	ITALY	EN	158	158	176	183	171	176	167	167	130	136	141	138	163	168	145	145	159	159	179	186	141	182	160	160
TUMOUR	INDIA	FT	158	158	171	171	169	169	164	174	134	134	130	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	INDIA	FN	158	158	177	189	165	168	0	0	130	130	143	145	0	0	121	126	191	193	179	179	196	192	138	158
TUMOUR	INDIA	GT	158	158	171	171	169	169	164	174	134	134	130	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	INDIA	GN	158	158	183	183	168	182	183	183	136	130	162	162	162	167	123	143	186	198	179	179	192	192	158	160
TUMOUR	INDIA	HT	158	158	171	171	163	169	164	174	134	134	132	134	152	154	123	123	178	178	179	179	140	140	145	154
HOST	INDIA	HN	158	158	165	183	172	165	168	168	136	130	147	130	171	156	121	129	185	185	179	181	182	139	148	160
TUMOUR	INDIA	IT	158	158	171	171	163	169	164	174	134	134	132	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	INDIA	IN	158	158	183	191	162	162	170	183	136	136	141	129	169	162	133	141	186	189	179	179	0	0	166	158
TUMOUR	AFRICA	LT	158	158	171	171	169	169	164	174	134	134	132	134	152	159	123	123	178	178	179	182	136	136	145	145
HOST	AFRICA	LN	158	158	177	177	170	172	0	0	128	130	136	138	175	171	141	134	193	189	179	179	141	141	160	160
TUMOUR	AFRICA	MT	158	158	171	171	163	163	164	174	134	134	132	134	152	158	123	123	178	178	174	174	140	140	145	145
HOST	AFRICA	MN	158	158	176	183	182	172	164	164	130	130	0	0	150	169	134	141	0	0	183	181	174	190	160	164
TUMOUR	ITALY	NT	158	158	171	171	163	169	164	174	134	134	132	134	152	158	123	123	178	178	179	179	140	140	145	145
HOST	ITALY	NN	158	158	183	187	0	0	168	179	132	138	138	138	177	165	140	140	159	159	173	181	182	160	165	158
TUMOUR	ITALY	OT	158	158	171	171	163	169	164	174	134	135	132	134	152	158	123	123	178	178	182	182	140	140	145	145
HOST	ITALY	ON	158	158	0	0	164	172	185	168	130	130	136	129	168	165	121	145	171	189	179	179	159	162	158	158
TUMOUR	ITALY	PT	158	158	171	171	169	169	164	164	130	134	132	134	152	154	123	123	178	178	182	182	140	140	145	159
HOST	ITALY	PN	158	158	160	183	180	180	0	0	0	0	145	138	0	0	145	145	159	159	179	179	137	137	0	0
TUMOUR	TURKEY	2	158	158	171	171	163	163	164	174	134	134	127	127	152	158	123	123	178	178	178	178	140	140	145	145
TUMOUR	TURKEY	3	158	158	171	171	163	169	164	174	130	134	127	134	152	158	123	123	178	178	178	178	0	0	145	145
TUMOUR	TURKEY	4	158	158	171	171	163	169	164	174	130	134	132	132	152	158	123	123	178	178	0	0	140	140	133	145
TUMOUR	TURKEY	5	158	158	171	171	163	163	0	0	130	134	132	132	152	158	123	123	178	178	178	178	140	140	145	145
TUMOUR	TURKEY	6	158	158	171	171	169	169	164	174	134	134	132	132	152	152	123	123	178	178	181	181	138	140	133	145
TUMOUR	TURKEY	8	158	158	173	173	163	169	164	174	134	134	132	132	152	152	123	123	178	178	178	178	140	140	133	145
TUMOUR	TURKEY	11	158	158	171	171	163	169	164	174	134	134	132	134	152	152	123	123	178	178	0	0	140	140	145	145
TUMOUR	SPAIN	77	158	158	171	171	169	169	164	174	134	134	132	134	152	152	123	123	178	178	0	0	140	140	145	145
TUMOUR	USA	301	158	158	171	171	169	169	164	174	130	134	0	0	0	0	123	123	178	178	181	181	138	138	145	145
TUMOUR	SPAIN	85	158	158	171	171	159	169	164	174	130	134	132	134	152	158	123	123	178	178	181	181	140	140	145	145
TUMOUR	ITALY	83	158	158	171	171	0	0	164	174	130	130	129	134	152	158	123	123	160	160	178	178	0	0	145	145
TUMOUR	ITALY	92	158	158	171	171	163	163	0	0	0	0	0	0	152	158	123	123	178	178	178	181	138	140	145	145
TUMOUR	ITALY	76	158	158	171	171	163	163	164	174	134	134	132	134	152	158	123	123	160	178	178	181	138	140	145	145

Samples	Origin	Name	93	93	H2	H2	H6	H6	H5	H5	125	125	H9	H9	2004	2004	2010	2010	2054	2054	2001	2001
TUMOUR	ITALY	AT	120	120	94	94	121	121	112	112	96	96	138	140	316	316	221	221	0	0	0	0
HOST	ITALY	AN	122	122	98	107	131	125	116	116	102	91	143	143	243	316	233	233	144	153	131	131
TUMOUR	ITALY	BT	120	120	94	94	124	124	112	112	96	98	138	140	317	317	221	233	168	168	0	0
HOST	ITALY	BN	122	124	96	106	131	125	112	116	102	98	151	151	234	238	225	299	169	153	139	131
TUMOUR	ITALY	CT	120	120	94	94	121	121	114	114	96	98	138	140	316	316	221	233	169	165	0	0
HOST	ITALY	CN	122	124	98	107	121	119	116	116	104	102	142	155	234	290	228	236	172	156	148	131
TUMOUR	ITALY	DT	120	120	94	94	121	121	112	112	96	98	138	140	316	316	221	233	168	168	0	0
HOST	ITALY	DN	122	133	96	106	121	119	112	114	102	108	142	142	242	246	236	241	169	153	148	144
TUMOUR	ITALY	ET	120	120	94	94	121	121	112	112	96	98	138	140	316	316	221	221	168	165	0	0
HOST	ITALY	EN	122	22	98	96	123	113	110	116	88	93	151	155	238	238	228	233	0	0	148	131
TUMOUR	INDIA	FT	120	120	94	94	121	121	112	112	94	96	138	140	234	234	221	233	157	157	0	0
HOST	INDIA	FN	137	135	100	102	119	123	114	114	91	98	145	157	230	242	228	236	169	172	148	131
TUMOUR	INDIA	GT	120	120	94	94	121	121	112	112	94	96	138	140	234	234	221	233	157	157	0	0
HOST	INDIA	GN	135	135	98	98	131	131	116	116	98	98	151	157	234	234	225	237	160	169	144	144
TUMOUR	INDIA	HT	120	120	94	94	121	121	112	112	94	94	138	140	308	308	221	233	152	156	0	0
HOST	INDIA	HN	122	130	96	109	128	119	116	112	86	92	151	157	243	317	236	236	148	173	144	139
TUMOUR	INDIA	IT	120	120	94	94	121	121	112	112	94	96	138	140	254	254	221	233	157	157	0	0
HOST	INDIA	IN	135	133	96	102	130	130	114	114	102	104	142	145	234	238	225	236	169	172	153	135
TUMOUR	AFRICA	LT	120	120	94	94	124	124	112	112	96	96	138	138	300	300	221	233	148	157	0	0
HOST	AFRICA	LN	122	122	103	107	127	121	118	113	88	94	151	151	243	243	237	237	153	169	148	131
TUMOUR	AFRICA	MT	120	120	94	94	124	124	112	112	94	96	0	0	308	308	221	233	161	161	0	0
HOST	AFRICA	MN	122	123	96	106	128	119	118	116	98	104	140	142	243	243	237	237	144	176	153	140
TUMOUR	ITALY	NT	120	120	93	93	124	124														
HOST	ITALY	NN	122	122	98	102	118	125														
TUMOUR	ITALY	OT	120	120	94	94	121	121														
HOST	ITALY	ON	130	122	96	98	116	119														
TUMOUR	ITALY	PT	120	120	93	93	124	124														
HOST	ITALY	PN	122	128	102	107	0	0														
TUMOUR	TURKEY	2	120	120	94	94	121	121														
TUMOUR	TURKEY	3	120	120	89	89	121	121														
TUMOUR	TURKEY	4	120	120	94	93	124	124														
TUMOUR	TURKEY	5	120	120	94	94	121	121														
TUMOUR	TURKEY	6	120	120	94	94	121	121														
TUMOUR	TURKEY	8	120	120	94	94	121	121														
TUMOUR	TURKEY	11	120	120	94	94	121	121														
TUMOUR	SPAIN	77	120	120	89	89	121	121														
TUMOUR	USA	301	120	120	94	94	121	121														
TUMOUR	SPAIN	85	120	120	89	89	121	121														
TUMOUR	ITALY	83	120	120	94	94	121	121														
TUMOUR	ITALY	92	120	120	94	94	121	121														
TUMOUR	ITALY	76	120	120	94	94	121	121														

Chapter 5

CTVT DLA Transcriptional analysis

5.1 Introduction

According to the immunosurveillance hypothesis of cancer the immune system protects the host against the development of tumours (Dunn et al., 2004a). The Major Histocompatibility Complex antigens play a key role in the recognition of tumour cells by the immune cells (Garrido and Algarra, 2001). Therefore defects in processing and presentation of MHC antigens represent an advantage for tumour progression. Loss of MHC antigen expression can occur at the genetic, transcriptional and post-transcriptional level (Algarra et al., 1999; Cabrera et al., 2003). Several studies have shown that many human and animal tumours do not express or down regulate the expression of MHC class I and II antigens (Algarra et al., 1999; Cabrera et al., 2003). Tumour cells obtain this result by deleting or mutating the MHC genes or several genes involved in the antigen presenting process, or by down regulating these genes by genetic and epigenetic mechanisms (Algarra et al., 1999; Nie et al., 2001; Cabrera et al., 2003). One of the mechanisms that reduces MHC class I expression involves β2 microglobulin expression (Garrido and Algarra, 2001). The loss or reduced expression of β2 microglobulin can be the result of homozygous or heterozygous mutations.

Early studies have shown that CTVT can be transplanted into immunocompetent animals of other canine species such as coyotes, jackals and foxes (Ajello, 1980; Cohen et al., 1984; Cohen, 1985). It has been reported that CTVT does not express β2 microglobulin on the surface of the tumour cells (Cohen et al., 1984). From this study it was suggested that the lack of β2 microglobulin could account for CTVT allotransplantability. Other studies have shown that naturally and experimentally

transplanted CTVT do not express DLA class I and II antigens during the progressive stage and that these antigens are expressed during the regression stage (Yang et al., 1987; Perez et al., 1998). A recent study shows that during progressive growth secretion of tumour growth factor beta (TGF- β1) by CTVT is a potent inhibitor of the local host immune response (Hsiao et al., 2004). Numerous studies suggest that in cancer heritable gene function alteration may be mediated by epigenetic as well as by genetic mechanisms. DNA hypermethylation and/or histone deacetylation contribute to the absent or down-regulated expression of different components of the tumour recognition complex (Magner et al., 2000; Magner and Tomasi, 2000).

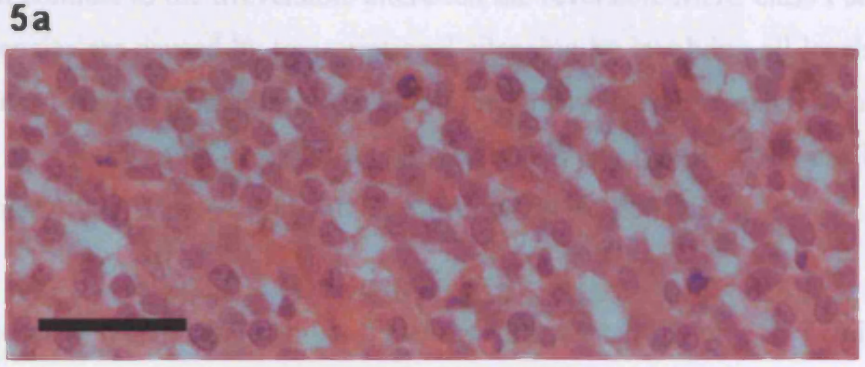
This chapter describes the transcriptional analysis of tumour DLA class I (DLA-88) and class II (DLA- DRB1, DQB1) genes by reverse transcriptase PCR (RT-PCR) of a tumour biopsy collected from a dog bearing a progressively growing naturally occurring tumour.

5.2 Results

In order to investigate if the reported lack of the MHC class I and II expression during the progression stage is due to transciptional or post transcription alterations, the mRNA expression level of Class I antigen (DLA-88) and Class II antigens (DRB1) was analysed. From DLA genotyping data tumour and host specific DLA-88 and DRB1 primers were designed in order to analyse specifically the differential expression of these mRNAs, between tumour and host in the same biopsy sample. Given that the MHC expression may be altered in an *in vitro* system (tissue culture of tumour cells), the RNA was extracted from a tumour biopsy (Dog C see Table 2.1 and Fig 2.1 c-d).

According to the tumour size and the clinical history of Dog C, the tumour was classified as a progressing tumour. Histopathological examination by haematoxylin and eosin stained tissue sections confirmed the clinical diagnosis and showed that the tumour cells represent over 90 % of the tumour tissue (Fig 5a).

Considering that the majority of tumour tissue is constituted of tumour cells, RT-PCR analysis shows (Fig 5b) that both the tumour DLA-88 alleles (α β) are down regulated with respect to the host stromal cells DLA-88 expression (allele 29 and 42). In contrast to host DLA-88 alleles, tumour alleles exibits a different level of expression, with a more pronounced downregulation in allele β. Fig 5b also shows that although host DLA DRB1 alleles were expressed, the DRB1 mRNA in tumour cells was undetectable. These results suggest that the transient expression of the tumour MHC Class I and II antigens during the progressive and regressive stage is due mainly to transcriptional silencing of the DLA genes rather than genes involved in transport and processing.



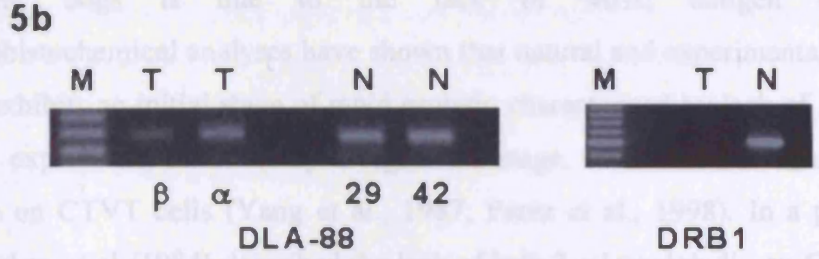


Fig 5a: Penile CTVT of dog C: Haematoxylin and eosin stained 4 µm section. Bar = 50 µm.

Fig 5b: Expression of DLA class I (DLA-88) and class II (DRB1) in the tumour of dog C by RT-PCR using tumour cell specific primers (T) and primers for the alleles of the host stromal cells (N).

5.3 Discussion

The immune system plays an important role in controlling the emergence or elimination of cancer cells . A wide range of immune mechanisms are involved in protecting the host against tumour cells. However, tumour cells acquire different mechanisms to escape immunological surveillance (Dunn et al., 2004a). Therefore, the immune system not only protects the host against development of primary cancers but also during tumour progression sculpts tumour immunogenicity by selecting tumours having an escape phenotype (Khong and Restifo, 2002; Dunn et al., 2004b). One of the most frequently described mechanisms of immune evasion is the partial or total lack of MHC antigen expression. The MHC deficiencies may be irreversible or reversible. The irreversible include mutations affecting the expression or function of MHC antigens and or proteins involved in MHC assembly and transporting, such as beta 2 microglobulin and TAP proteins (Garrido and Algarra, 2001). In contrast to the irreversible alteration the reversible MHC class I and class II deficiencies are caused by transcriptional silencing by involving all levels of the MHC class I-restricted antigen presentation machinery, and their expression can be restored by cytokines or by DNA demethylation/histone hyperacetylation (Magner and Tomasi, 2000; Moreau et al., 2003; Morimoto et al., 2004).

Early and more recent studies suggest that the transplantability of CTVT in immune competent dogs is due to the lack of MHC antigen expression. Immunohistochemical analyses have shown that natural and experimentally induced CTVT exhibits an initial stage of rapid growth, characterised by lack of MHC class I and II expression, followed by a regressive stage, where the MHC antigens are detected on CTVT cells (Yang et al., 1987; Perez et al., 1998). In a preliminary study Cohen et al (1984) described the lack of beta 2 microglobulin on CTVT cells, so suggesting that the MHC class I deficiency observed in CTVT is due to beta 2 microglobulin alterations (Cohen et al., 1984). However my transcriptional analysis indicates that reversible expression of the DLA class I and II antigens is due mainly to their transcriptional silencing.

Epigenetic modifications such as DNA methylation and histone deacetylation play a major role in cancer through transcriptional silencing not only in tumour suppressor genes but also in genes involved in immune evasion (Esteller and Herman, 2002). Recent studies have highlighted the importance of epigenetic modifications by the observation of the hyper methylation of the MHC promoter sequences in MHC deficient tumours in absence of mutations in MHC genes and beta 2 microglobulin. Methylation can also inhibit indirectly MHC transcription by silencing the MHC transactivator such as coactivator class II transactivator (CIITA) and or genes involved in assembly and transport (Nie et al., 2001; Morimoto et al., 2004). Although the reduced or absent levels of MHC class I antigens helps tumour cells to evade the classical T cell dependent immune responses, it simultaneously activates the natural killer cells by the 'missing self' signals (Miller, 2001).

Recent findings have suggested that tumour cells can exploit the mechanism adopted by the semi-allogeneic fetus in controlling the maternal immune response (Miller, 2001). Therefore the fetal trophoblast-maternal relationship has been suggested to be analogous to a tumor-host interaction (Rouas-Freiss et al., 2005). Like tumour cells the trophoblast does not induce transplantation immunity and resists natural killer (NK) cells. It has been suggested that trophoblast does not induce transplantation immunity because it does not express classical polymorphic MHC genes, whereas it does express a non-polymorphic Class I molecules HLA-G (Hunt et al., 2005). This plays an important role in protecting the fetus from maternal immune attack by the interaction of inhibitor receptors expressed in maternal natural killer and T cells.

Canine MHC Class I include only one classical Class I gene (DLA-88), and several non classical genes such as DLA-79, DLA-64, DLA-12 (Bryan et al., 1997; Burnett et al., 1997; Graumann et al., 1998). However, to date no detailed studies have been undertaken to elucidate their function in pregnancy and in cancer. Given their

reduced genetic polymorphism, the differential transcription analysis between tumour and host cannot be assessed using specific PCR primers. Thus a systematic analysis of several tumours during different stages of growth and regression using laser capture microdissected tumour cells would be required to test the involvement of non classical MHC genes in evading the NK cell response.

One mechanism of cancer immune evasion is the local suppression of anti-tumour immunity. Several studies have suggested that tumour cells can directly secrete several cytokines or indirectly induce the stromal cells to secrete inhibiting factors in order to evade the immune responses or to induce an immuno-tolerance status. Recently Hsiao et al (2004) in experimentally transplanted CTVT have shown that CTVT cells secrete tumour growth factor beta (TGF-β 1), thus inhibiting the host immune response. Several studies have observed that in immunocompetent dogs CTVT is localised exclusively at the superficial mucosae and that intravenous inoculation of tumour cells trigger an efficient immune response able to destroy tumour cells (Cohen, 1985). This suggests that CTVT cells are able to evade the local mucosal immune response. However the majority of the immunogical studies regarding CTVT have analysed the systemic immune response in vivo and by in vitro assays.

In contrast to alpha and beta T cells, gamma/delta T cells are more abundant in epithelial and mucosal tissues (Hayday, 2000). Several findings have suggested that gamma/delta T cells are involved in modulation of the immune responses in tumours and in several infectious and parasitic diseases and tumour (Kapp et al., 2004). Recently it has been reported that epithelial gamma/delta cells are capable of recognising the antigen expressed by damaged or stressed keratinocytes and producing keratinocyte growth factors (KGFs) and chemokines, therefore arguing for their active role in wound repair (Jameson et al., 2002). These observations suggest that the local immune response should be thoroughly analysed in order to elucidate the immune evasion mechanisms adopted by CTVT.

Chapter 6

SRY Amplification

6.1 Introduction

The ability to discriminate whether a biological sample originated from a male or a female is essential in forensic science. Over the years using polymerase chain reaction methods, different DNA markers on the sex chromosomes have been shown to be useful in gender identifications (Butler, 2001). This ability has been also used to discriminate the sexual origin in different sex mismatched conditions like organ and haematopoietic transplantation and fetal-maternal chimerism (Aractingi et al., 1998; Wang et al., 2002).

One of the most frequently used DNA markers within the male Y chromosome is the sex-determining region (SRY). The canine Y chromosome is the smallest chromosome and the SRY region is located in the non-recombinant region (Olivier et al., 1999). In dogs several PCR based assays have been used to study naturally occurring inherited disorders of testicular differentiation (Meyers-Wallen et al., 1995), and to monitor the chimerism in bone marrow transplantation (Fiegler et al., 2002). In monkeys, it has been shown that PCR assays based on SRY amplification can detect less than four cells in the presence of 10^5 background cells (Sieverkropp et al., 2005).

The transmission of a cancer cell is a rare complication of human tissue and organ transplantation (Penn, 1997). Recently the SRY marker has been successfully used to demonstrate the donor cell origin of Kaposi's sarcoma cell in a female post-transplant recipient (Barozzi et al., 2003). Given that CTVT is a sexually transmitted tumour, and given the success of the sex DNA typing approach in

resolving the origin of donor cells in a sex mismatched recipient, this approach could be applied to CTVT although it has not previously been used to test the cellular transmission of CTVT. In this study, 9 CTVT samples collected from female hosts were analysed for the presence of the Y chromosome DNA by PCR using Y chromosome specific primers.

6.2 Results

The presence of the Y chromosome DNA in the tumour cells was tested by PCR amplification of a 104 bp segment of the Sex Region of the Y chromosome (SRY). Among the 9 tumour samples derived from female dogs the matched normal tissues were also examined from 3 animals (blood of Dogs, E, H and I). In primary amplification no clear SRY bands were observed in the tumour samples (pictures not shown). In contrast, the SRY sequence was efficiently amplified from DNA taken from the blood of a healthy male dog. Therefore a second, nested amplification was tested to visualize the specific 104 bp band. Of the 9 tumour samples, 8 were positive, and one was negative (sample 30) (Fig 7.1 a and b). Where matched tumour and host DNA was analysed each of the 3 fresh tumour samples were positive, but the matched host blood samples were negative (Fig 7.1a). To exclude the possibility of PCR contamination, a second region of the Y chromosome called the 650 region was also tested. Due to the length of this region (650 bp), only the DNA extracted from fresh samples could be analysed (Dogs E, H and I). A specific 650 bp band was amplified only in the tumour (a faint band is present in sample HT) and not in the matched host samples (Fig 7.2).

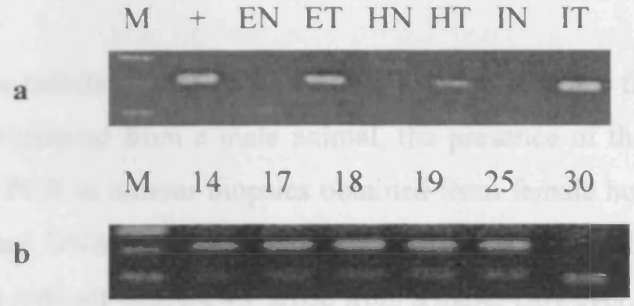


Fig 7.1a and **7.1b**: Detection of Y chromosomal DNA by SRY PCR in tumour tissues from female hosts. M, 50 bp DNA ladder, + positive control (peripheral blood mononuclear cell DNA from a male host). Lanes EN and ET are matched normal and tumour samples respectively from dog E; lanes HN and HT matched normal and tumour samples from dog H; lanes IN and IT matched normal and tumour samples from dog I; Lanes 14-30 paraffin embedded tumour samples.

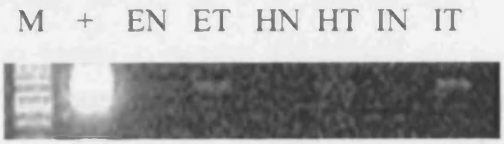


Fig 7.2: Amplification of 650 bp fragment of Y chromosome in tumour samples from female hosts. M, 50 bp DNA ladder, + positive control (peripheral blood mononuclear cell DNA from a male host). Lanes EN and ET matched normal and tumour samples from dog E; lanes HN and HT matched normal and tumour samples from dog H; lanes IN and IT matched normal and tumour samples from dog I.

6.3 Discussion

To further test the cellular transmission of CTVT and to evaluate the possibility that the CTVT has originated from a male animal, the presence of the Y chromosome was analysed by PCR in tumour biopsies obtained from female hosts. The presence of Y chromosomal DNA in the tumours and not in the normal blood of matched female hosts may indicate that CTVT arose from a male. However, despite using the same amount of template in the primary PCR as for the positive male control, the tumour samples exhibited a much lower intensity of the PCR bands than in the control samples.

Although unlikely, these results might be explained by assuming that mutations in the flanking region of both the SRY and the 650 regions could decrease the PCR amplification efficiency. Alternatively the presence of the Y chromosome DNA in female hosts might occur if male fetal progenitor cells traffic to the mother during pregnancy and persist in the maternal circulation for many years (Jimenez et al., 2005). However this hypothesis is ruled out by the absence of Y chromosomal DNA in the matched female blood samples.

Cytogenetic analyses of tumours in human and animals have reported that in tumours with a high degree of chromosome rearrangement, the Y chromosome is more frequently lost (Kujawski et al., 2004). Given that CTVT is highly aneuploid, the Y chromosome could have been lost from a male tumour at an early stage of the CTVT progression, before the stabilization of its abnormal karyotype. If only a small proportion of tumour cells retained the Y chromosome that could explain the weak signal. I did not study whether the X chromosome was diploid or haploid.

An alternative explanation is that the detection of the Y chromosomal DNA in sexually transmitted CTVT tumours could come from passenger cells seeded during

intercourse by the previous male host. This could be from sequestered sperm cells, although this seems unlikely in CTVT samples taken weeks after the presumed date of sexual exposure, or it could be from male stromal cell in the tumours.

Generally tumour development is viewed as an autonomous process, in which, the accumulation of somatic mutations have led cancer cells to overcome the microenvironmental control to gain independence from the host. However recent evidence has highlighted the importance of the stromal cells in cancer initiation, progression and in invasion (Kiaris et al., 2004; Zigrino et al., 2005).

Tumour stroma contains a rich cellular population, which includes fibroblasts, smooth muscle cells, endothelial cells and inflammatory cells. Tumour cells interact with stromal cells and the microenvironment through the release of soluble factors and by cell-cell and cell-matrix contact. It is thought that tumour cells alter stromal cell gene expression, by the production of specific factors such as growth factors and cytokines, thus shaping the microenvironment surrounding the tumour. However recent analysis of stromal cells in breast tumours have identified specific mutations in tumour suppressor genes, suggesting that stromal cells can play an active role in tumorigenesis. Recent *in vivo* studies using mouse models and xenografts suggest that mutations in stromal fibroblasts can also initiate epithelial tumours (Bhowmick et al., 2004). It has been further suggested that chronic inflammation might lead to an alteration of stromal cell gene expression profiles which decrease their negative control over the cell proliferation, thus favouring the development of cancer (Coussens and Werb, 2002).

It can be argued that the presence of CTVT-associated stromal cells during transplantation could stimulate the host immune response therefore eliciting the tumour rejection. However, recently it has been shown that in addition to MHC down-regulation, the CTVT cells induce local immunosuppression to overcome

immune rejection (Hsiao et al., 2004). Thus it can be argued that local immune suppression might also favour the proliferation of normal passenger cells.

New blood vessel formation (angiogenesis) is a fundamental event in the process of tumour growth and metastasis. New blood vessels can derive from the pre-existing vasculature or by direct acquirement of progenitor cells (Carmeliet, 2003).

Although tumour transplantation has been widely used since the time of Novinski (1876) as a tool to study and propagate tumour cells *in vivo*, the role of the stromal cells (passenger cells) in tumour growth is only just beginning to emerge. In a recent study Duda et al (2004) evaluated the fate of the tumour associated endothelial cells, demonstrating that endothelial cells proliferating in the new host remain functionally active for more than 4 weeks after transplantation, and govern the initial tumour neo-vasculature (Duda et al., 2004).

The important role of stromal cells has also been demonstrated in transplantation, where it is claimed that they are responsible for the prolongation of allograft survival (Ikehara, 2001) . Therefore the presence of passenger cells during natural transplantation of CTVT could favour the transplantation and proliferation process.

The presence of Y chromosome DNA in tumour tissue derived from a female host needs further analysis, to determine whether the Y chromosome is present inside or outside the tumour cells themselves. For this reason CTVT chromosome spreads prepared using fresh tumour biopsies will be analysed by Fluorescence In situ Hybridisation (FISH) in collaboration with Professor Malcolm A. Ferguson-Smith and Dr David Sargan at the Cambridge Resource Centre for Comparative Genomics, Centre for Veterinary Science, University of Cambridge. If the Y chromosome is present outside the tumour cells, the morphology of the 'male' cells will be important to determine their origin. In contrast, if the Y chromosome is inside of the CTVT cells, specific Y chromosome microsatellites markers can be analysed for

phylogenetic studies as counterpart of the autosomal microsatellites and the mt-DNA analysis that it has been undertaken in this study.

Chapter 7

Breed of origin and age of CTVT

7.1 Introduction

The place and origin of the domestic dog has long been debated. Charles Darwin argued that the phenotypic variability observed in dogs is due to multiple and independent origins from two or more canine species. Konrad Lorenz suggested that dogs posses phenotypic traits of both wolves and jackals (Wayne and Ostrander, 1999). However, molecular genetic studies based on mitochondrial control region sequences from wolves, jackals, coyotes and dogs have shown that wolves are the sole ancestors of the domestic dogs and that modern domestic dogs originated from at least four maternal wolf lines (Wayne et al., 1997; Savolainen et al., 2002). A recent phylogenetic study based on the examination of the mt-DNA control region from 654 dogs representing all major dog populations worldwide, showed that dog populations originated from a single genetic pool arguing for a East Asiatic domestication event about 15,000 years ago (Savolainen et al., 2002).

Although mitochondrial DNA analysis has been successfully used to elucidate the phylogenetic relationship between dogs and wolves, its evolution rate is too low to allow the inference of genetic relationships between dog breeds (Vila et al., 1999b; Savolainen et al., 2002). To date there are more than four hundred breeds with high phenotypic diversity. The majority of the dog breeds have had a recent origin from a diverse founding stock with subsequent interbreeding between breeds (Sutter and Ostrander, 2004). Mitochondrial DNA analysis indicated a low correlation between mt-DNA haplotype and breed; therefore the same haplotype can be found in different breeds, and different haplotypes are present within a breed (Savolainen et

al., 2002). In order to clarify the genetic relationship between dogs several studies based on the Dog Leukocyte Antigens (DLA) analysis have shown a high variation between breeds and relatively low intra-breed variation of DLA class II haplotypes (Kennedy et al., 2002). Thanks to the important advance in theoretical population genetics, microsatellites have been used to infer population structure and demographic history in several species, including humans and dogs. A study based on 10 nuclear microsatellite DNA markers and involving 250 dogs belonging to five different breeds, showed that modern individual clustering methods allow the correct assignment of individuals to their breed of origin (Koskinen, 2003). Recently Parker et al (2004), in an attempt to analyse the genetic relationship between breeds, analysed 414 dogs belonging to 85 dog breeds, using 96 dinucleotide microsatellite markers; they showed that microsatellite markers combined with phylogenetic and clustering analysis can assign 99% of individual dogs to their original breed (Parker et al., 2004).

Tumours can be described as populations of tumour cells, originating from a single transformed cell, evolved through successions of selection and clonal expansion (Greaves, 2002). Despite of numerous applications of microsatellite analysis in cancer, such as molecular markers to analyse the monoclonal origin, or the tumour response to chemotherapy or immuno-therapy or detect genetic abnormalities, they have only recently been used to infer genetic histories in mutator tumours (Tsao et al., 2000). Given that the mutation rates in most tumours are less than 10^{-6} per locus per division, the detection of somatic mutations in non-mutator type tumours is difficult (Shibata, 2001).

The inference of the time of the most recent common ancestor (TMRCA) is based on the assumption of common mutation rates between populations (Stumpf and Goldstein, 2001). For tumours, this issue is difficult to resolve, because tumours with mutator phenotypes during progression may have different mutation rates between tumour populations.

The results described in the Chapter 4 showed that CTVT arose from a common ancestral neoplastic cell of a single host. Although CTVT cells are highly aneuploid, the tumour genotype is remarkably stable. Phylogenetic analysis has been widely used to understand past events in different human and animal populations, and it can similarly be used to understand the origin and evolution of CTVT.

This chapter describes inferences of the phylogenetic origin and an estimate of the age of CTVT. First the DLA alleles specific to CTVT were examined to gain an indication of the breed of origin. Second a more intensive analysis of tumour genotypes was carried out based on a set of dinucleotide microsatellite markers used previously to determine genetic relationships among 85 different dog breeds. Third, mt-DNA tumour haplotypes were analysed using different phylogenetic methods.

7.2 Results

7.2.1 Breed of origin according to DLA haplotype

DRB1 polymorphisms have been extensively used in genetic and evolutionary studies in several canid species (Kennedy et al., 2002). In contrast, DLA-88 polymorphisms are poorly characterised. Therefore a phylogenetic analysis was performed using host and tumour allele sequences found in this study and previously published DRB1 sequences including alleles of dogs, North American and European grey wolves and coyote.

The phylogenetic analysis was performed using the maximum likelihood method implemented in PAUP. The ML tree (Fig 7.1) shows that the CTVT DRB1 alleles are clustered with nine DRB1 sequences; however DRB1 04101 and DRB1 04201 described in North American wolves and coyotes are closer in term of nucleotide

substitutions. Of these nine DRB1 haplotypes, the 04701 allele has been found in association with the DQA1 00402 allele in Huskies.

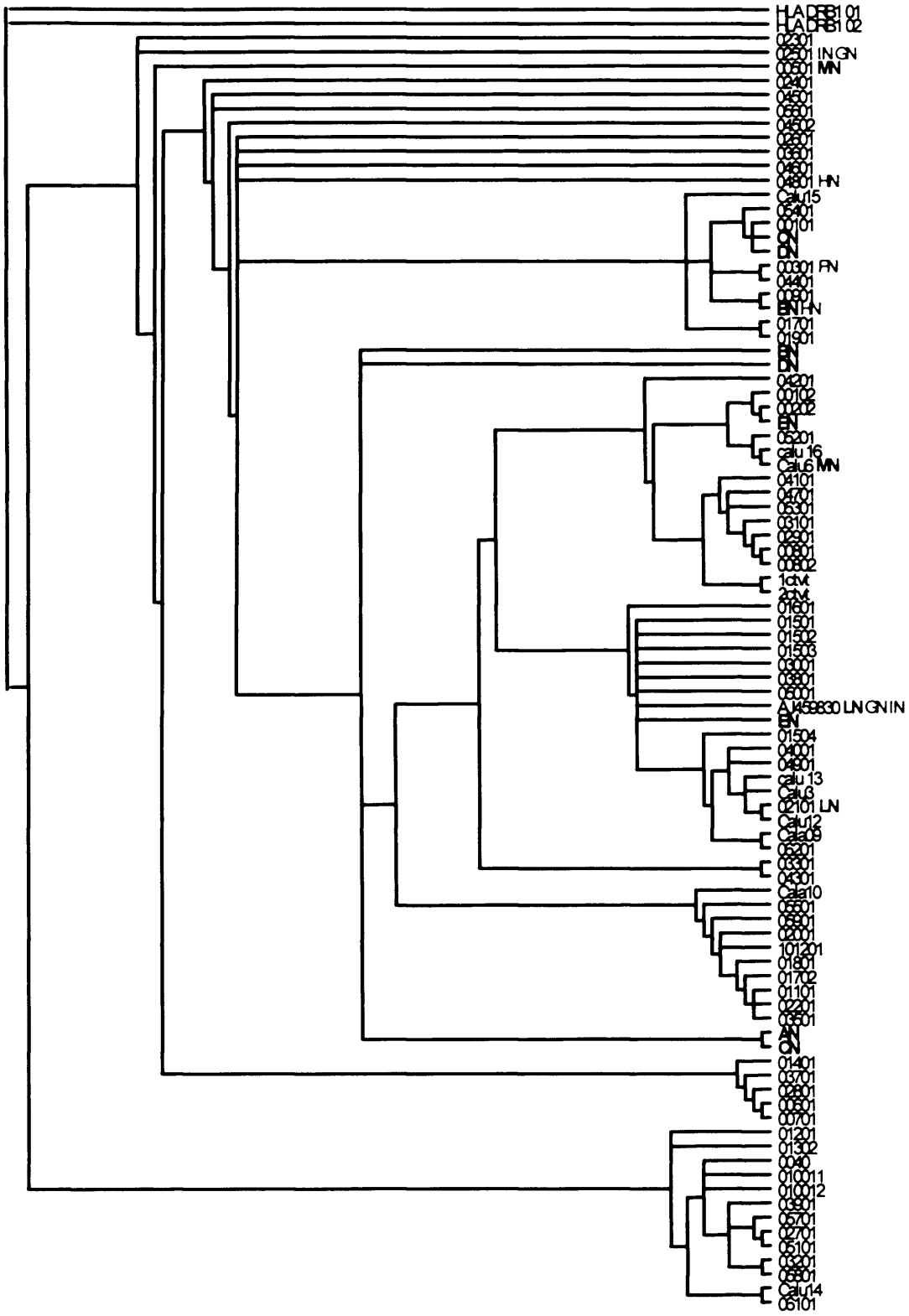


Fig 7.1. DLA DRB1 phylogenetic relationship between tumours and their hosts and published DRB1 alleles of dog, wolves, and coyotes. Human DRB1 alleles were used as outgroup. Alleles in Blue, Green and Pale Blue represent host alleles found in Italy, Kenya and India respectively. Tumour alleles are indicated in red.

7.2.2 Breed of origin according to microsatellites

In order to determine the origin of the CTVT in more detail, seventy-three microsatellite loci were genotyped in three matched tumour and normal samples one each from India, Italy and Kenya. A further analysis of twenty-four microdissected tumour samples of diverse geographic origin was performed using a subset of 18 of the 73 markers. These microsatellites markers were chosen because they have been previously genotyped by Parker et al (2004) in a set of 8 wolves from different countries and 414 dogs representing 85 different breeds. Tumour genotypes were compared with the genotypes described by Parker, using a common positive control sample to ensure consistent conversion of peak sizes to alleles (binning) between runs.

A model-based clustering algorithm, Structure (Pritchard et al, 2000) was used to investigate the relationship between CTVT and the data obtained from dogs belonging to 85 breed and 8 wolves. The Structure method groups individuals in to a pre-specified number of clusters, as explained in Chapter 4 for comparing tumours and hosts. In this case no prior information regarding the breed of origin was applied to the algorithm. As shown in (Fig 7.2), using 18 dinucleotide markers and assuming 2 clusters (K=2), the tumour samples were clustered with wolves and a set of dog breeds that were previously grouped by Parker et al (2004) in an ancient breed cluster, because they are genetically most similar to wolves. This finding is consistent with the breed assignment based on DLA alleles. At higher values of K, all tumours were grouped in into a single cluster that is distinct from both dogs and wolves, thus confirming the monoclonal origin of the CTVT.

To determine the specific origin of CTVT, additional clustering analysis was performed, focusing on the subset of individuals, that shows genetic similarity to CTVT (Fig 7.3). The model used allowed CTVT samples to have a mixed ancestry, so that if the ancestor was a mongrel dog, then CTVT ancestry should be spread across two or more breeds. In addition, to force the tumour samples to cluster with dog/wolf cluster(s), the PFROMPOPFLAGONLY was used. In this analysis, all

individuals from Parker et al (2004) were correctly assigned to their respective original breed, whereas tumours were clustered more strongly with wolves, suggesting that CTVT possibly originated in wolves. Given that the wolf sample is larger than the dog samples (8 individuals versus 5), in order to check if this biases the clustering of tumour samples with wolves, a set of 3 wolves was randomly dropped to rerun the structure analysis with the same parameters. In this case the posterior assigment of tumour to the wolf cluster was slightly lower (at K= 4 from 0.74 to 0.66).

A different additional analysis was performed using the Neighbour-Joining method, to build an unrooted tree based on the pairwise distance among genotypes (nonparametric clustering method). This analysis (Fig 7.4 a) again indicates the similarity between CTVT and wolves. The relationship between CTVT and wolves was confirmed when the tree was constructed using all dog and wolf populations (Fig 7.4 b).

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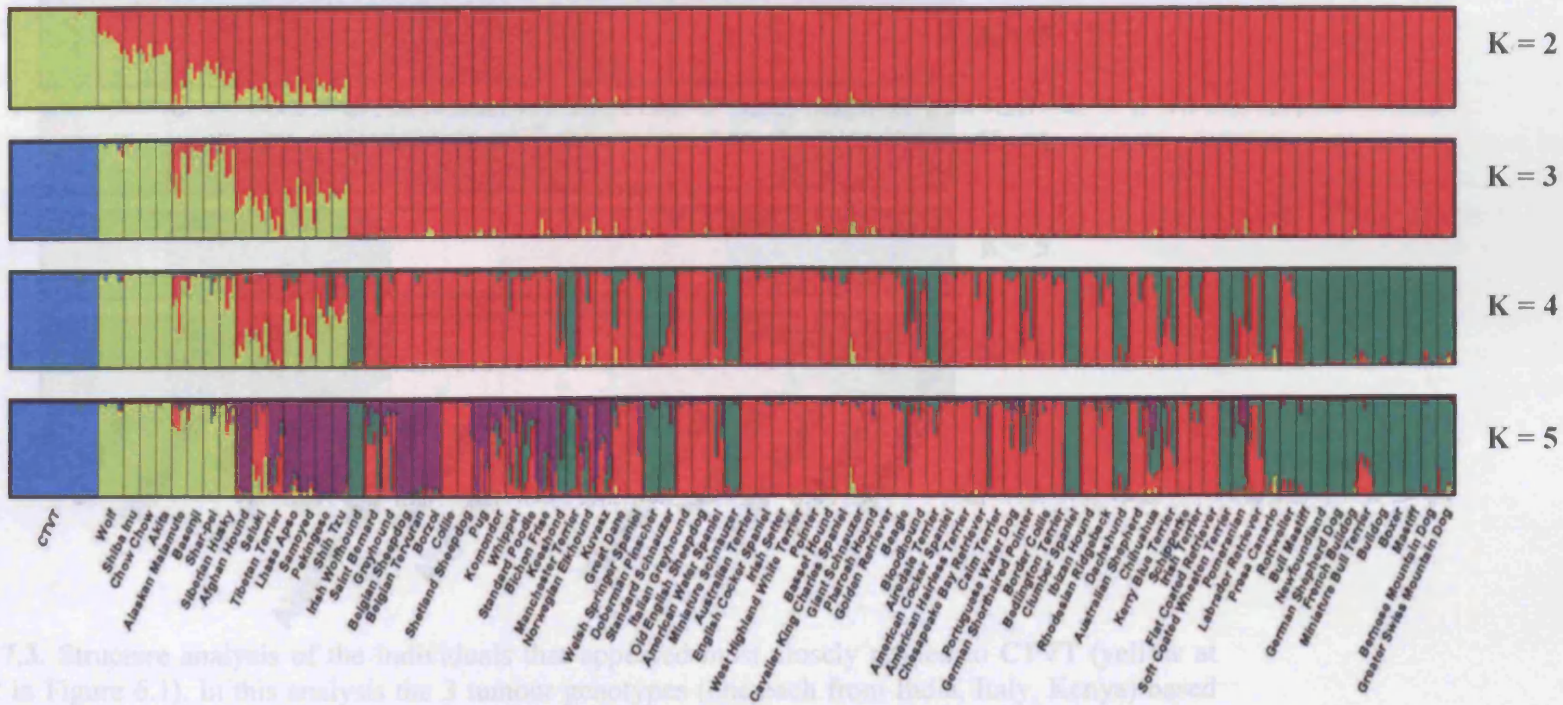


Figure 7.2. Structure analysis of the relationship between tumor genotypes and normal genotypes represented by 414 dogs of different breeds and 8 wolves, based on 73 microsatellite loci. Each panel shows the results from a model-based clustering algorithm, Structure, that assigns sampled individuals, based on their genotypes, to a pre-specified number K of clusters. Each tumor sample, or individual, is represented by a vertical line, with colored segments indicating the proportion of that individual's membership in each cluster. At K=2, the tumours cluster clearly with wolves and certain dog breeds; for larger K values, the tumors form a distinct group, indicating a common origin.

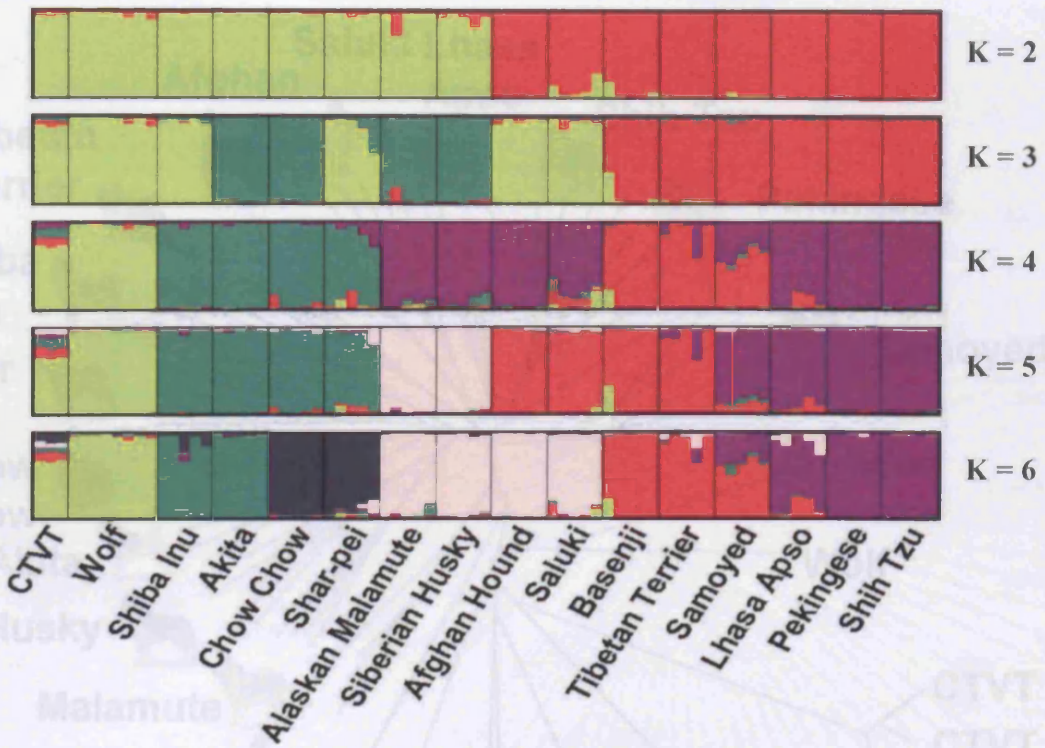


Fig 7.3. Structure analysis of the individuals that appeared most closely related to CTVT (yellow at K=2 in Figure 6.1). In this analysis the 3 tumour genotypes (one each from India, Italy, Kenya) based on 73 dinucleotide markers, were forced to cluster to dog/wolf clusters without creating their own cluster. Structure runs were performed using an admixture model, with correlated frequency assuming different K values (2-6).

a

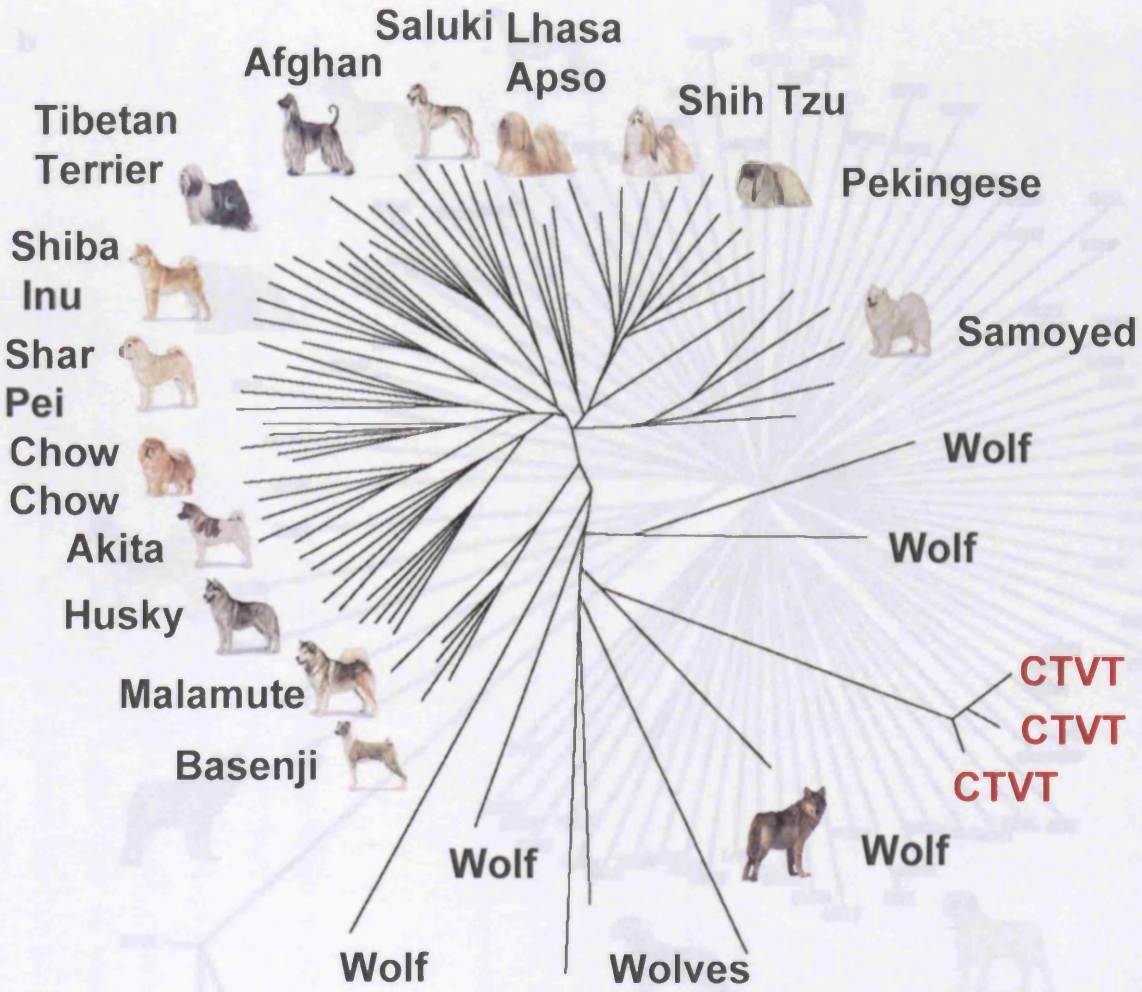
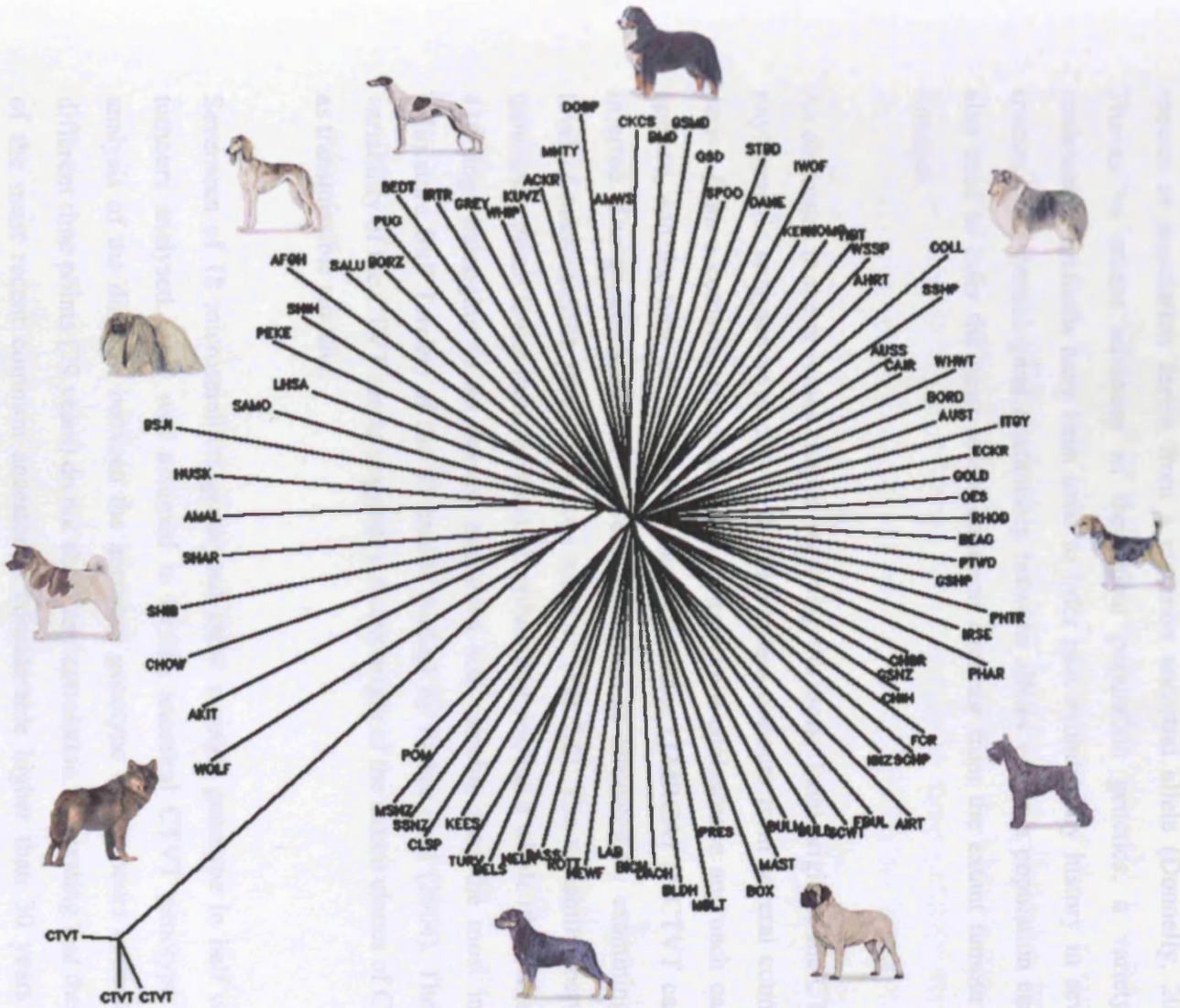


Fig 7.4 Neighbor-Joining trees based on pairwise distance matrix obtained analyzing 3 CTVT samples (India, Italy, Kenya) with **a**, wolves and dogs that were clustered with CTVT sample using Structure analysis (Fig 6.2) and **b** (next page), with all 85 dog breeds and wolf population, where all individuals within a breed were considered as a single population.

b


DOBP
CKCS
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GSD
STBD
IWOF
BEDT
BTR
GREY
KUVZ
ACKR
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DANE
PUG
KEMRCOMP
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IRSE
CHOW
GSMR
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KEES
AIRT
CLSP
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EBUL
TURV
BELS
NELM
MASS
LAB
MAST
RDTT
NEWF
BMDM
DACH
BLDH
BOX
MBLT
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CTVT CTVT

7.2.3 Age of the CTVT

According to Coalescent theory, the extant alleles (DNA polymorphism) within a species or population derive from a common ancestral allele (Donnelly, 2001). Thanks to recent advances in theoretical population genetics, a variety of coalescence methods have been used to infer past evolutionary history in several species. This genealogical relationship between alleles within a population can be also used to infer the most recent common ancestor from the extant tumour cell lineages.

As discussed in the previous chapter regarding the monoclonal origin of the CTVT, phylogenetic analyses have shown that the extant tumours from several countries derive from a common ancestral clone. Therefore a coalescence approach can be applied, and the time of the most common ancestor (TMRCA) of CTVT can be inferred. The genetic variation between tumours was estimated by examining the level of microsatellite variation across tumours (Fig 7.5). The variability between tumours is much lower than variation of wolves and dogs as a whole (8 wolves and 414 dogs belonging to 85 breeds) and even less variable than the most inbred (Miniature Bull Terrier) of the 85 breeds studied by Parker et al (2004). The low variability of the CTVT seems suggests a recent origin of the extant clones of CTVT as transmissible parasite.

Seventeen of 18 microsatellites genotyped show a single genotype in half of the tumours analysed. This was assumed to be the ancestral CTVT genotype. The analysis of the distance between the ancestral genotype and tumours collected at different time points (29 years) do not show any correlation, suggesting that the time of the most recent common ancestor is considerable higher than 30 years. The microsatellite variation analysis expressed as average expected heterozygosity (Fig 7.5) shows a lower variability within CTVTs than within dogs in general and as a

single breed, and within wolves. Although there is no direct information about the microsatellite mutation rate of CTVT, the genotyping results show a genetic stability at dinucleotide repeats, indicating that CTVT has an efficient mismatch repair mechanism.

Given the unclear estimates of the microsatellite mutation rate in the canid family, a mutation rate of 10^{-3} to 10^{-4} per generation for typical loci, reported in humans was used (Huang et al., 2002) in order to estimate the time since divergence from a common tumour ancestral lineage. Assuming the absence of back mutation (a small effect when most genotypes are identical), the probability that a genotype matches the ancestral genotype is exp(-mu t), where mu is the mutation rate, per genotype per year, and where t is the time in years since the common ancestor. If we suppose that genotype mutation rates of 10^{-3} to 10^{-4} /year are appropriate for CTVT, an age estimate ranging from 250 to 2500 years is obtained.

A further estimate of the CTVT age was computed using the mt-DNA control region. A 580bp sequence obtained from 11 fresh specimens, and a shorter 257bp fragment obtained from 21 micro-dissected tumour cells from paraffin embedded specimens were analysed. In order to place our data in the context of dog and wolf mt-DNA sequences, we analysed our data along with additional sequences obtained from previous studies of canid (Tsuda et al., 1997; Vila et al., 1997; Randi, 2002; Savolainen et al., 2002).

Two maximum likelihood trees were computed using the program PAUP* and analysing a subset of the tumour mt-DNA sequences (21 tumour haplotypes) and the previously obtained dog and wolf sequences (45 haplotypes). As noted before (Savolainen et al, 2002), there is considerable rate heterogeneity across sites within the sequence. This fact combined with the small number of substitutions separating most of the sequences, means that there is considerable uncertainty in the estimated tree. Nonetheless, the tumours again fall into two main groups within clade A

(Figures 7.7. and 7.8). The first maximum likelihood tree (Fig 7.7) was constructed by assuming that the tumour sequences are monophyletic. It is noteworthy that as noted in chapter 4, the tumours form two distinct clusters that correspond to haploid and diploid status at DQA1. Given that the branch lengths are proportional to the maximum likelihood times elapsed on each branch, this tree suggests that the common ancestor age of the haploid and diploid CTVT clades are each only slightly younger than the origin of dog clade A.

The second maximum likelihood tree (Fig 7.8) was constructed without assuming that the tumour sequences are monophyletic. This tree indicated that two tumour sequences 8C and 5F group separately from the main tumour clades. These tumour haplotypes are both short sequences obtained from fixed samples, and so there may be uncertainty about their true position on the tree. Moreover, contamination by host mt-DNA cannot be excluded. To estimate the time of origin of CTVT, the genetic variability within CTVT mt-DNA haplotypes relative to dog mt-DNA in general was determined (Fig 7.6). Savolainen et al. (2002) have previously estimated that the most recent common ancestor of dog clade A occurred about 41,000 years ago, assuming a unique single origin and a star-shaped genealogy (Savolainen et al., 2002). Fig 7.6 shows that pairwise variability within CTVT is actually larger than the variability within dog Clade A (1.38% vs. 0.73% pairwise divergence). Within the two subclades defined by DQA1 status, pairwise variability is lower, but still comparable with that of the entire clade A (0.84% and 0.69% for the haploid and diploid clades respectively). Hence, on their own, the mt-DNA data argue for an extremely ancient origin of CTVT, roughly coincident with the domestication of dogs.

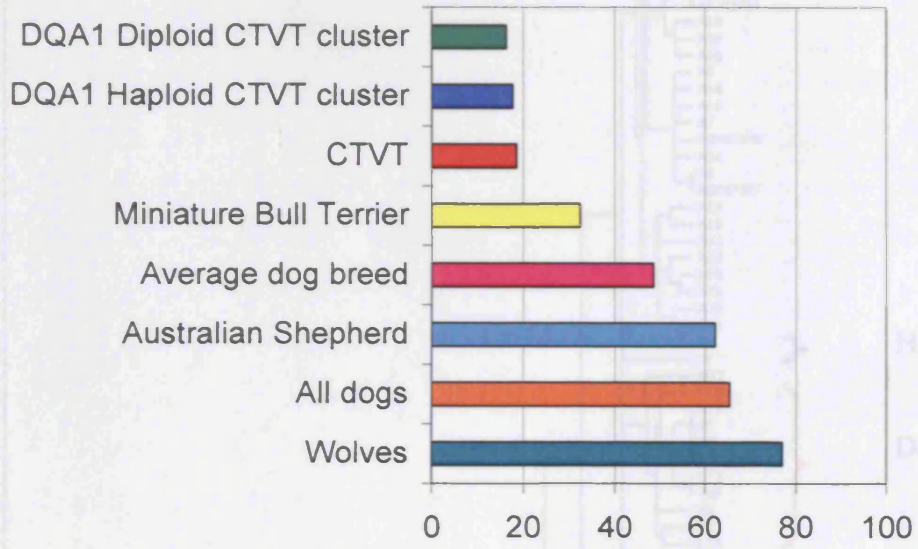

Fig 7.5 Microsatellite average expected heterozygosity Miniature Bull terrier and Australian Sheperd represent the most and least heterozygous dog breeds respectively across 85 dog breeds analyzed.

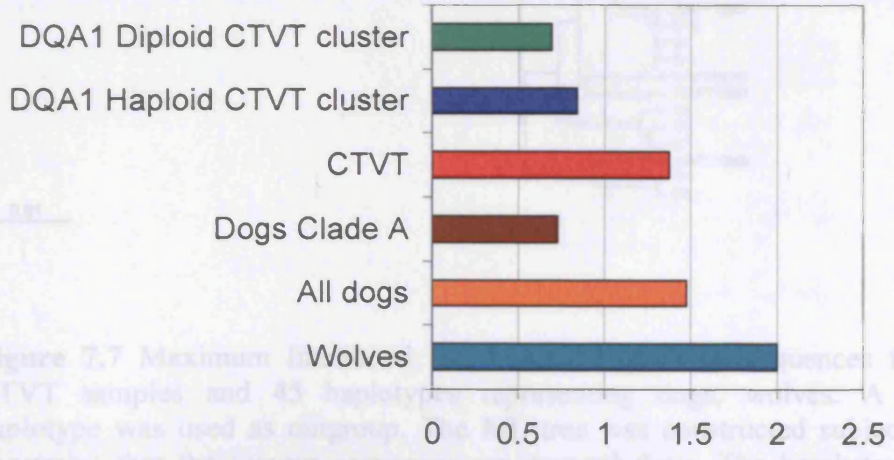

Fig 7.6: mt-DNA pairwise sequence divergence

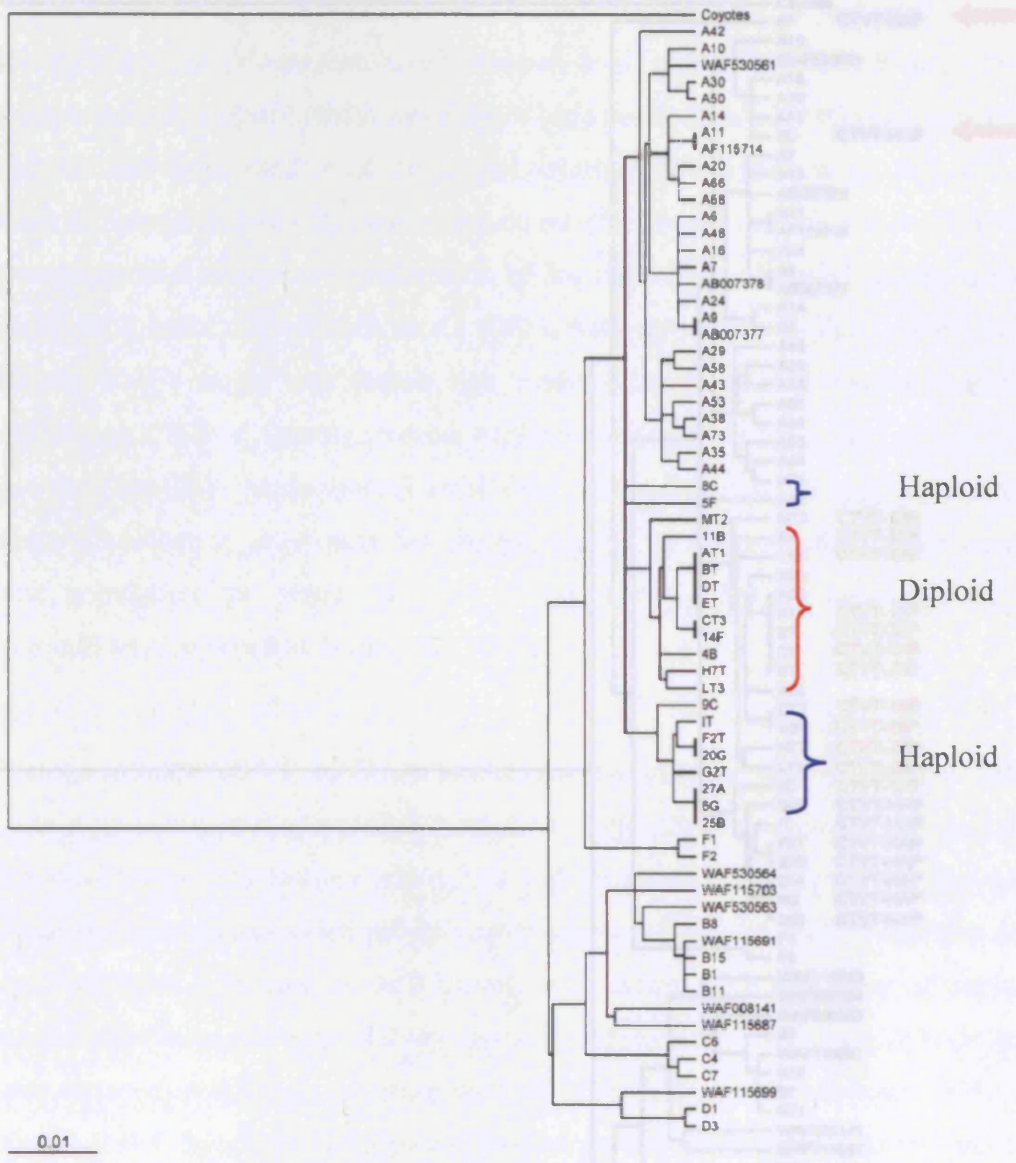

Figure 7.7 Maximum likelihood (ML) tree of mt-DNA sequences from 21 CTVT samples and 45 haplotypes representing dogs, wolves. A Coyote haplotype was used as outgroup. The ML tree was constructed subject to the constraint that the tumour sequences are monophyletic. The brackets indicate tumours samples with a haploid or diploid DQA1 status. The red arrow indicates tumour haplotypes separated from the main two clusters.

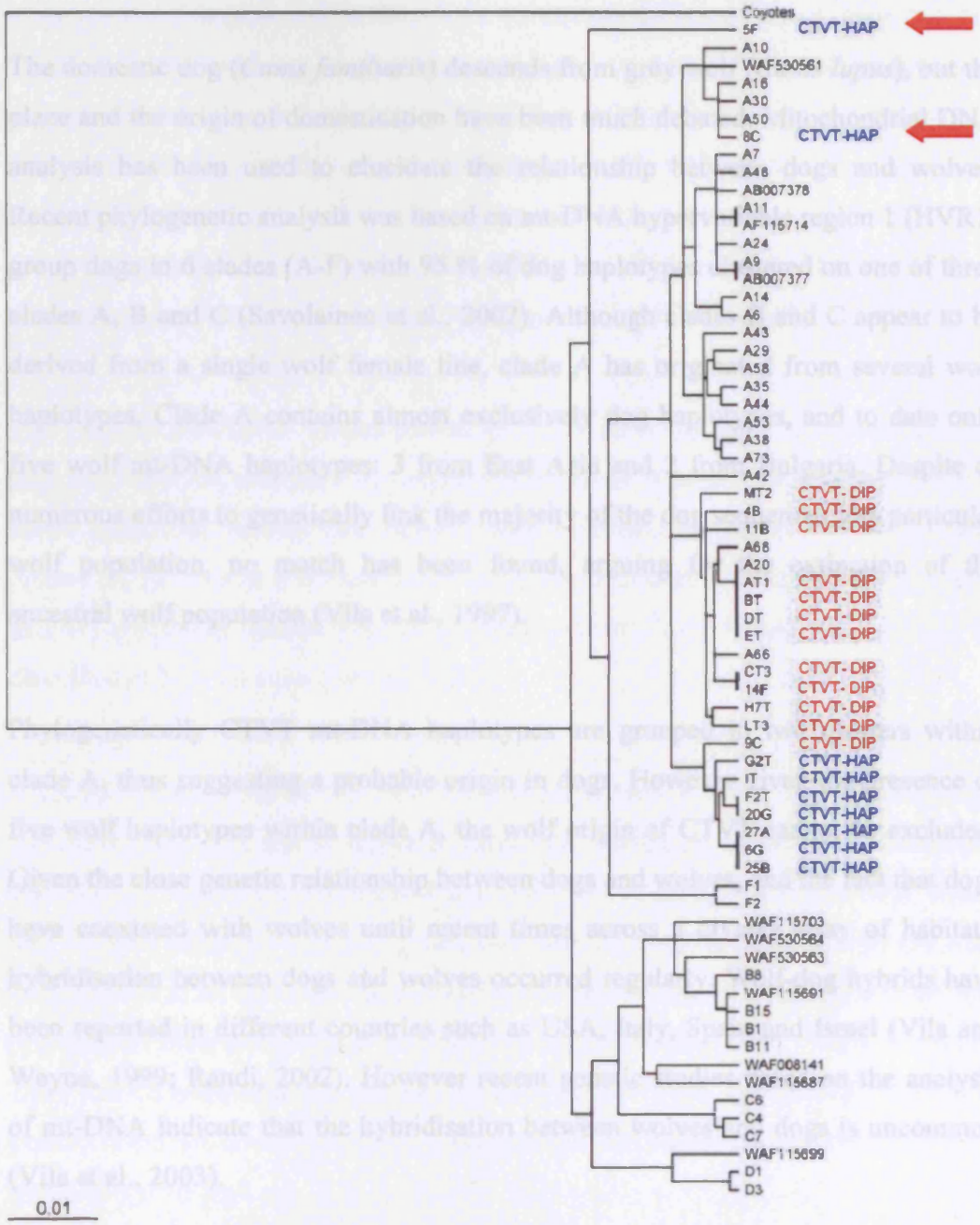

Figure 7.8 Maximum likelihood (ML) tree of mt-DNA sequences from 21 CTVT samples and 45 haplotypes representing dogs, wolves. A Coyote haplotype was used as outgroup. The ML tree was constructed without assuming that the tumor sequences are monophyletic. The brackets indicate tumours samples with DQA1 haploid and diploid status. Red arrow indicates CTVT samples separated from the main tumour clades.

7.3 Discussion

The domestic dog (*Canis familiaris*) descends from grey wolf (*Canis lupus*), but the place and the origin of domestication have been much debated. Mitochondrial DNA analysis has been used to elucidate the relationship between dogs and wolves. Recent phylogenetic analysis was based on mt-DNA hypervariable region 1 (HVR1) group dogs in 6 clades (A-F) with 95 % of dog haplotypes clustered on one of three clades A, B and C (Savolainen et al., 2002). Although clades B and C appear to be derived from a single wolf female line, clade A has originated from several wolf haplotypes. Clade A contains almost exclusively dog haplotypes, and to date only five wolf mt-DNA haplotypes: 3 from East Asia and 2 from Bulgaria. Despite of numerous efforts to genetically link the majority of the dog sequences to a particular wolf population, no match has been found, arguing for the extinction of the ancestral wolf population (Vila et al., 1997).

Phylogenetically CTVT mt-DNA haplotypes are grouped in two clusters within clade A, thus suggesting a probable origin in dogs. However given the presence of five wolf haplotypes within clade A, the wolf origin of CTVT cannot be excluded. Given the close genetic relationship between dogs and wolves, and the fact that dogs have coexisted with wolves until recent times across a diverse array of habitats, hybridisation between dogs and wolves occurred regularly. Wolf-dog hybrids have been reported in different countries such as USA, Italy, Spain and Israel (Vila and Wayne, 1999; Randi, 2002). However recent genetic studies based on the analysis of mt-DNA indicate that the hybridisation between wolves and dogs is uncommon (Vila et al., 2003).

These observations raise the possibility that CTVT may have originated from a wolf-dog hybrid. However, the homozygosity present in the most polymorphic DLA class II genes suggests that both parents of the original CTVT host were related.

A genetic comparison of the canine MHC class II genes between wolves, dogs and coyotes, shows that several DLA class II alleles are shared between these species (Seddon and Ellegren, 2002). Despite a recent domestication event from wolves, substantial allele sharing has been observed only at the DQA1 locus. It has been argued that the reduction of shared DRB1 and DQB1 alleles between wolves and dogs may be due to the severe reduction of the wolf population size, with consequent genetic drift and loss of alleles, in conjunction with a large increase in the dog population size. Thus the DLA DRB1 and DQB1 alleles observed in dogs would have been present in ancestral wolf populations (Seddon and Ellegren, 2002).

The DLA-88 and DRB1 alleles present in CTVT have not been so far identified in any canid species, although the tumour DRB1 allele is close to alleles described in North American wolves and coyotes and Alaskan huskies. The CTVT DQB1 present in the tumour has been described in North American and European wolves and in several dog breeds. The DQA1 allele present in the tumour has been so far described only in Huskies (allele 00402).

A recent investigation of the DLA class II haplotypes in over one thousand dogs representing 85 pure breeds shows an extensive inter-breed, but minimal intra-breed variation. It was also demonstrated that the level of homozygosity is high within breed for all three DLA class II genes, but only dogs belonging to rare breeds were homozygous at all three loci. In contrast to purebred dogs, feral dogs and large wolf populations show a high level of heterozygosity (Kennedy et al., 2002). Therefore DLA class II homozygosity present in CTVT indicates that the original CTVT host derived from an inbred population, possibly an isolated breed belonging to Spitz group or a wolf pack.

Because wolves live in packs that are primarily family units, there is considerable opportunity for incestuous mating. Yet some studies suggest that within wolf packs, mated wolves are rarely related as siblings or as parent-offspring (Seddon and

Ellegren, 2004). This observation suggests that in general, wolf packs are established by unrelated or more distantly related wolves. Therefore despite of frequent opportunity, incestuous reproductive succession is not a common means to attain reproductive success. However several genetic studies suggest that incestuous mating in wolves occurs primarily where wolves are prevented from out-breeding, such as a population bottleneck or geographical isolation (Seddon and Ellegren, 2004).

Savolainen et al. (2002) have shown from the comparison of the mt-DNA control region in more than 100 dog breeds that there is low correlation between mt-DNA haplotype and breeds. Therefore although the mt-DNA analysis may resolve the difference between species, it cannot assign an individual at a specific breed (Savolainen et al., 2002). Phylogenetically CTVT tumours are grouped in two clusters within clade A. In cluster 1 there are tumours that share their haplotypes with a previous described haplotypes named A18 found in different breeds, whereas cluster 2 seems to be derived from haplotypes A22 also found in different breeds.
Given that wolves are highly mobile and that up to few centuries ago were widespread throughout the Holarctic, the mitochondrial DNA shows a low geographical structure (Vila et al., 1999a). In contrast, some dog haplotypes show a correlation with their geographical origin. However the haplotypes A18-A22 are present in breeds of different geographical origin.

Interestingly tumours within cluster 1 show a diploid DQA1 status, whereas tumours in cluster 2 posses a haploid DQA1 status. The DQA1 deletion present in the cluster 2 seems to indicate that cluster 1 is the original CTVT mt-DNA haplotype. However, the ancestral tumour haplotype may be extinct.

The inferred ancestral haplotype is genetically close to haplotype A31 that has been found only in West Laika dogs (a breed belonging to the Spitz group).

Although more than one haplotype was found in some tumours, which were grouped in the same cluster, no tumour possessing both haplotype clusters (1 and 2) was found. Recently it has been shown that mt-DNA heteroplasmy is present both in tumour and normal cells, and that a mutated mt-DNA molecule may reach a homoplasmic status in a normal cell in seven days (Mambo et al., 2003). It has been suggested that homoplasmy occurs due to the selective advantage of the mutated haplotype (Bianchi et al., 2001). However theoretical studies have demonstrated that genetic drift could also explain this phenomenon (Bianchi et al., 2001; Coller et al., 2001).

In contrast to mt-DNA analysis, microsatellites have been successfully used to assign dogs to their original population or breed. Phylogenetic analysis based on microsatellites showed divergence between breeds of ancient origin that are closer to wolves, and breeds of presumed modern European origin (Parker et al., 2004). Recent studies aimed to analyse the differentiation and hybridisation between wolves and dogs using microsatellites markers have showed that wolves from different populations are more related compared to dogs of different breeds (Randi, 2002).

Genotyping analysis of microsatellites indicates that CTVT has originated in wolves. However, some caution is required due to the small sample sizes of each breed, and the fact that the available dog data were limited to pedigree breeds (Parker et al., 2004), so an origin in domestic dogs is not excluded. Moreover, the Structure assignment analysis indicates also that CTVT forms its own cluster. This might be due to the fact that CTVT derives from an unsampled wolf or dog population, or that during evolution CTVT has diverged from its original population. In contrast to wolf and dog populations as a whole, CTVT shows low microsatellite diversity. Generally the reduced microsatellite variability is due to several factor such as genetic isolation, bottleneck, or inbreeding (Ellegren et al., 1996). Although the mechanism of mutation in microsatellites is due to slip-strand

mispairing during replication, a further mechanism that increases genetic variability is recombination between homologous chromosomes during meiosis (Ellegren et al., 1996). The lower genetic variability of CTVT may be due to selection or, given that CTVT reproduces asexually, by the absence of recombination.

Development of cancer in humans is due to accumulation and selection of different mutations in at least six cancer associated genes (Hahn and Weinberg, 2002a). Given that tumours evolve within a finite lifetime, mutator mutations that accelerate the mutation rate are thought to contribute frequently to the carcinogenesis (Loeb and Loeb, 2000). However the reduced microsatellite diversity founded in CTVT indicates that CTVT is evolving with a normal mutation rate, thus suggesting a recent origin of the tumour.

Although an increased mutation rate may accelerate the accumulation of oncogenic mutation (positive clonal selection), it also affects other genes thus reducing the cellular fitness. These mutations may mediate the selective elimination (negative clonal selections) of mutator clones (Beckman and Loeb, 2005). Recent theoretical studies have indicated the existence of a mutation rate limit beyond which the negative clonal selection limits the survival of mutator clones (Sole and Deisboeck, 2004; Beckman and Loeb, 2005).

Although the extant CTVTs are genetically stable, the remarkable constant chromosomal rearragments suggests that in the early stages of its evolution, CTVT exhibited chromosomal instability (CIN). Genetic defects in DNA damage repair genes have been associated with a variety of predisposition syndromes, such as hereditary non-polyposis colorectal carcinoma (HNPCC), Bloom Syndrome and ataxia-telengiectasia, and also with other tumours that possess a mutator phenotype, characterised by an high mutation rates, and consequently by genetic instability (Charames and Bapat, 2003).

Recent studies indicate that besides the genetic defects, epigenetic mechanisms are also responsible in promoting the genetic instability by altering the genetic expression of DNA repair genes (Charames and Bapat, 2003). The epigenetic mechanisms involve both loss and gain of DNA methylation as well as alteration of histone modifications (acetylation and phosphorylation). Although epigenetic mechanisms can occur at any time during tumour progression they occur more frequently at the early stage of tumour development (Baylin and Ohm, 2006). In contrast to genetic alteration, epigenetic alteration can be reversed. From the above consideration, it can be argued that epigenetic mechanisms can be responsible for the genetic stabilization of CTVT. Therefore understanding how CTVT has stabilized its genome may have biological and therapeutic implications for cancer.

Although cancer cells die when their host dies, the advent of the mammalian cell culture and cloning allowed the development of immortalized cell lines able to live and spread in laboratory cultures. Human HeLa cells were cloned by Puck in 1952 and since then are continually propagating (Weiss, 2005).

Novinsky made the first description of CTVT in 1876 in Petersburg. However a few years later CTVT has been described in several countries, thus suggesting an older emergence date. The impossibility to analyse ancient tumours makes difficult to calibrate a molecular clock for CTVT. However assuming a stable mutation rate of 10^{-3} to 10^{-4} (at least since its emergence as a parasite) the genetic divergence of the dinucleotide repeats indicates that the tumour arose between 250 and 2500 years ago. Assuming that the tumour acquired the parasitic features from the begining, this date represents the time the tumour first arose. In contrast if the tumour has evolved as parasite in different hosts, this date could represent a later bottleneck, where the ancestral tumour parasite clone arose.

Savolainen et al (2002) using a substitution rate of 7.1 % per million years, and assumed a divergence time of one million years between coyotes and wolf, to

suggest an approximate age of 41,000 ± 4000 years for clade A, assuming a single origin

In contrast to microsatellites, assuming a normal substitution rate, the mt-DNA data argue for an extremely ancient origin of CTVT, roughly coincident with 41,000 years. These contrasting results may be due to the fact that the mt-DNA mutation rate in CTVT is higher than normal. Thus in CTVT the somatic mutations in mt-DNA do not associate with nuclear microsatellite instability.

Given that the mitochondrial genome does not posses its own repair mechanisms, nuclear genes are responsible in repairing the mt-DNA mutations (Bianchi et al., 2001). In contrast to the nuclear genome, mt-DNA is more susceptible to electrophile and oxidant damage leading to rapid accumulation of homoplasmic mutations (Mambo et al., 2003). From the above considerations the estimated of the age of the most recent common ancestor of CTVT obtained by microsatellites seems to be more likely. Although the estimated date of between 250 and 2500 years represents a recent evolutionary origin, CTVT represent the oldest known immortalised somatic cell in continuous propagation, having undergone countless mitoses and animal to animal transfer since it arose in a progenitor.

Chapter 8

Conclusions

In vertebrates, genetic variation can be only inherited through germ cells, which are separated from somatic cells early in embryogenesis. It has been argued that this ontogenic separation of the germ line limits competition between somatic and germ cells (Ridley, 2004a). Buss has pointed out that this kind of development known, as "Weismannist" is exceptional among diverse multicellular organisms, including colonial organisms where the generation of new individuals can be formed from somatic cells. In these organisms, cellular competition allows the emergence of super competitor or parasitic cells capable of reproducing themselves and spreading 'infectiously' to the other member of the colony by fusion, thus forming a chimera (Buss, 1982).

Natural selection operates upon biological entities that can be divided into different levels of organization from genes through chromosomes, cells, organs, and organisms, to groups within a species, and between species. In contrast to asexual organisms where selection between somatic cells can take place, In vertebrates natural selection is limited to germ cells (Ridley, 2004a). Recent studies have demonstrated that in the colonial ascidian *Botryllus schlossery,* the unit of selection is the stem cell, which is able to produce both somatic and germ lineage cells. It has been also shown that these stem cell lineages may compete with stem cells of closely related colonies for access to gametic or blastogenic niches that will form a new individual (Laird et al., 2005).

In vertebrates the somatic cell lineages die when the organism dies, therefore limiting any form of selection to the germ lines that are transmitted by sex to form a new individual. Similarly somatic cancer cell lineages come to an end with the organism's death. CTVT thus represents a special case, because somatic cancer cells

have acquired the ability to spread naturally to other individuals acting like a parasite, thus, gaining independence from its original host.

In order to survive parasites evolve to increase their ability to propagate in the next host; thus the target of selection is transmission success (Frank, 1996; Weiss, 2002). Therefore a successful parasite may exploit its host resources in a prudent way without causing excessive damage, because a rapacious exploitation, although it increases the reproductive rate within a host, will reduce the probability of transmission among hosts. Furthermore, a limited resource may select the parasite for a prudent way of exploitation instead of a rapid and rapacious way because it will reduce the total resource for the future progeny. Competition between parasites for limited resources influences virulence and therefore transmissibility (Frank, 1996).

According to Kin Selection theory (Hamilton, 1964), cooperation between parasites (prudent exploitation of resources) is favoured by natural selection if the net reproductive value (total progeny in a susceptible population) among genetically related parasites is higher than the cost (reproductive value within the host). Therefore if genetically unrelated parasites use a limited resource, natural selection favours the parasite genotype that is capable of using a major quantity of resources to produce more progeny, thus increasing the conflict between parasites and the rapacious exploitation within a host (Frank, 1996). These observations suggest that individual selection favours short-term benefits while the kin selection favours long-term benefits.

According to natural selection theory, no selection can take place between genetically identical cells. The genetic relatedness between cells is influenced by many factors; among them one of the most important is the mutation rate (Michod, 1996). Cells of somatic tissues may accumulate mutations that are naturally selected. According to the clonal evolution theory of cancer, somatic cells bearing

mutations that increase the reproductive rates are expanded (Nowell, 1976; Greaves, 2002). While mutations in oncogenes and tumour suppressor genes enhance the reproductive rate, mutations in genetic instability genes increase mutation rates (Michor et al., 2003; Michor et al., 2004). Genetic instability is manifested as microsatellites instability (MSI) or chromosomal instability (CIN). Although enhanced mutation rates may slow down the growth rate due to the accumulation of lethal mutations, recent theoretical studies have demonstrated that genetic instability is a disadvantage only when clonal expansion is accelerated (Beckman and Loeb, 2005; Nowak et al., 2005).

Although the debate on whether genetic instability plays an important role during the early stages of a tumour progression or is just a late stage consequence of the cancer evolution is still open (Sieber et al., 2005), the results obtained in this study show that CTVT is genetically stable. However, cytogenetic studies suggest that genetic instability might have played an important role in the early stages of the CTVT evolution.

Is has been suggested that due to the absence of recombination, asexual organisms can suffer from an effective accumulation of irreversible deleterious mutations throughout time, leading the finite population to extinction (Muller, 1964), called Muller's ratchet. Although CTVT is an asexual reproducing cell it does not appear to be subject to Muller's ratchet. This could be explained by the fact that CTVT may be not old enough to be affected by Muller's ratchet (Gordo and Charlesworth, 2000). However it has been shown that the reduction in population size during the parasite transmission bottleneck, accelerates the ratchet (Bergstrom et al., 1999). In contrast, weak purifying selection can lead to the complete cessation of the ratchet (Gordo and Charlesworth, 2000).

It can be argued that similar factors may also play an important role in CTVT. Recently it has been proposed that cell-cell fusion between tumour and normal cells

or between tumour cells, might allow recombination between different genomes, thus contributing to cancer progression (Parris, 2006). However, in CTVT the constant rearranged karyotype and the genotyping results, suggest that recombination between different genomes is most unlikely to have played an important role during its evolution.

Another factor that influences relatedness between parasites is the bottleneck effect during the transmission event (the numbers of parasites that are transmitted from one host to the other). Therefore species with higher mutation rates are more prone to conflict than species with low mutation rates, and larger samples (number of parasites) during transmission decrease relatedness favouring competition between parasites (Bergstrom et al., 1999).

CTVT cells with their stabilized genome may reflect kinship selection and reduced virulence thus aiding host survival and onward tumour transmission. According to the theory of somatic evolution, tumour cells typically evolve towards the selfishness that ultimately results in a more malignant phenotype with severe effects on the host's fitness (Nunney, 1999; Greaves, 2002; Michor et al., 2004). In contrast for the evolution of the parasite, host conditions are extremely important, and virulence has to be modulated to limit host damage (Frank, 1996). CTVT is a sexually transmitted neoplastic disease; therefore the fitness of its host is essential for the tumour transmission, especially in a natural environment where the males compete to mate.

It is generally accepted that down-regulation of the MHC antigens represents the major mechanism for tumours to escape the immune surveillance system (Dunn et al., 2004a). Furthermore, it has been observed that MHC negative tumours tend to be more malignant (Kageshita et al., 1999). The lack of expression of MHC antigens in CTVT seems to be in contrast to the reduced pathogenicity of CTVT, because assuming the evolution of a tumour within an individual, selection would increase

tumour malignancy, thus decreasing host fitness (Frank, 1996). Therefore the within a host evolution is in conflict with the observed features of the CTVT, which have been selected to limit host damage. These parasite features may be explained assuming that CTVT has evolved through a multi-host evolutionary model. In an inbred population, the tumour could have been transplanted to a different host having the same DLA haplotype, so avoiding tumour rejection. In this first stage the tumour cells have been selected to maximise transplantability and transmission within a syngeneic population. Moreover, an infected female must survive and be fit enough to progress through a successful oestrous cycle in order to transmit the tumour to a new male.

The reproductive success of a parasite is correlated with the frequency of the encounters (susceptible hosts) present in the environment (Frank, 1996). Wolves are monogamous, where only the alpha male and females are allowed to breed, whereas dogs are more promiscuous. Therefore a successive step during CTVT evolution may have been to increase the range of susceptible hosts, from a syngeneic population to an allogeneic population. This process has been necessary because after tumour regression, the host is immune to CTVT reinfection so decreasing over a period of time the number of susceptible hosts.

It has been suggested that self/non-self recognition systems have evolved as defence against pathogens and somatic cell variants. From genetic studies applied to the cellular slime mould, *Dictyostelium mucoroides*, Buss proposed that the self/non-self recognition system has evolved to limit the spread of somatic or germ cell parasites in colonial organisms (Buss, 1982). It may be argued that the driving selection for the evolution of the MHC system and cell mediated adaptive immunity in early-jawed vertebrates may have been as much to protect against spreading cancer cells as against infections, because invasive and metastatic tumours develop only in vertebrates, whereas infections are universal.

In humans, cellular transmission of cancer has been observed in immuno-suppressed transplant recipients if the donor organ or tissue inadvertently contains occult tumour cells (Barozzi et al., 2003; MacKie et al., 2003). In contrast to the natural transmission of CTVT to immunocompetent dogs, the post-transplant tumour is not rejected because of the immunosuppressive treatment. However many tumours in humans down regulate the MHC class I and II expression (Garrido and Algarra, 2001; Khong and Restifo, 2002), and therefore MHC defective tumours might also proliferate in immunocompetent individuals. Similarly, the cellular transmission of MHC positive tumour cells, especially tumours of genital tract, could be facilitated among AIDS patients. Therefore the possibility exists for tumour cells to survive and proliferate in different hosts from their original. It is not clear why parasitic tumours have not emerged more frequently.

However, the natural transmissibility of CTVT may be not unique. From karyotype analysis Cooper et al (Cooper et al., 1964) reported that a transmissible hystiocytic tumour in inbred Syrian hamsters appears to be a case of cellular transmission. Recently, a tumour has been described in the Tasmanian devil called Devil Facial Tumour that based on cytogenetic analysis may also be a case of cellular transmission (Pearse and Swift, 2006). Recent genetic analysis of this endangered marsupial species has shown a very low degree of genetic diversity, which is consistent with a recent founder effect. It has been suggested that island effects and repeated periods of low population density may also have contributed to the low genetic variation within Tasmanian devil population (Jones et al., 2004). Although no MHC analysis has been performed, it can be argued that the Devil Facial Tumour may have emerged within a syngeneic population.

The foregoing discussion illustrates that CTVT represents a special case of somatic cell parasitism. As a transmissible tumour cell, it is not entirely unique if the Syrian hamster and Tasmanian devil tumours are accepted to originate from transmissible cells. However, such tumour outbreaks may be self-limiting in local populations,

such as hamsters within a single captive inbred colony, or Tasmanian devils within a small, partially inbred population. It is remarkable that as shown in this thesis, CTVT is a monoclonal tumour that has spread worldwide and has sustained transmission for at least 250 years, probably much longer.

In conclusion, CTVT is a most interesting biological phenomenon. It challenges us to think more deeply about genome stability in evolving tumour cell populations; how somatic cells may evade allograft rejection; and whether the evolution of adaptive cell-mediated immunity in vertebrates may have been driven in part by protection against allogeneic malignant cells.

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